University of Alberta

Drug Delivery to Osteoclast Receptor Targets

by

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DEDICATION

To my parents, Sai Gopal and Vidya, and my siblings, Rahul and Sameera, thank you for your unending and unconditional support.

ABSTRACT

Osteoporosis is a skeletal disorder that affects millions of people worldwide, and is characterized by the accelerated loss of bone mass. Current anti-resorptive drug approaches such as bisphosphonates and salmon calcitonin exhibit severe side effects and very low bioavailability, respectively. In this study, we have designed, synthesized, and performed preliminary tests on a novel conjugate that targets the RANK receptor on bone resorbing cells (osteoclasts) *in vitro* with one arm, while delivering a specific effector molecule, calcitonin, to osteoclasts with the other arm. First, we successfully generated osteoclasts from precursor RAW 264.7 cells and confirmed that they were functional. We also designed a resorption assay that can be used to test the efficacy of new and existing anti-resorptive drugs. RAW 264.7 cells were then treated with an antibody to RANK to prove that anti-RANK could be used as a targeting mechanism. We then showed that delivery of calcitonin-loaded anti-Calcitonin antibodies onto osteoclasts results in the association of calcitonin onto its receptors on osteoclasts. Finally, we constructed a novel conjugate: calcitonin–Streptavidin–anti-RANK, and showed that it can be used to introduce calcitonin into an osteoclast-like microenvironment.

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ACRONYMNS

IGF: Insulin Growth Factor
TGF-β: Transforming Growth Factor β
FGF: Fibroblast Growth Factor
BMP: Bone Morphogenic Protein
IgG: Immunoglobulin G
BMU: Bone Multicellular Unit
BRC: Bone Remodeling Compartment
OP: Osteoporosis
DEXA: Dual Energy X-ray Absorptiometry
qCT: Quantitative Computed Tomography
BMD: Bone Mineral Density
LVA: Lateral Vertebral
MRI: Magnetic Resonance Imaging
μCT: Micro-Computed Tomography
USPTF: United States Preventative Task Force
ISCD: International Society for Clinical Densitometry
BMI: Body Mass Index
LBM: Lean Body Mass
FM: Fat Mass
CFU-M: Colony-Forming Unit-Macrophage
M-CSF (or CSF-1): Macrophage Colony Stimulating Factor
RANKL: Receptor activator of Nuclear Factor κB Ligand
RANK: Receptor activator of Nuclear Factor κB
SFK: Src Family Kinases
PI3K: Phosphoinositide 3-kinase
RGD: Arginine – Glycine - Aspartic Acid
ITAM: Immunoreceptor tyrosine-based activation motif
DNAX: DNAX-activating protein 12
FcTγ: Fc-receptor common γ subunit
NFAT: Nuclear Factor Activator of T-cells
mTOR: mammalian Target Of Rapamycin
S6K: S6 kinase
PDK-1: Phosphoinositide-dependent kinase 1
TNFα: Tumor Necrosis Factor α
TNFR: Tumor Necrosis Factor Receptor
DC: Dendritic Cell
cDNA: complimentary/copy Deoxyribonucleic Acid
MMP: Matrix Metalloproteinase
OPG: Osteoprotegerin
mRNA: Messenger Ribonucleic Acid
TRAF: TNFR-associated factor
IKK: IκB kinase
MAPK: Mitogen-associated protein kinase
PLC: Phospholipase C
IP3: Inositol triphosphate
PKC: Protein Kinase C
IFN: Interferon
MFR: Macrophage Fusion Receptor
SIRP: Signal Regulatory Protein IgSF: Immunoglobulin Superfamily
DC-STAMP: Dendritic Cell-Specific Transmembrane Protein
TRAP: Tartrate-resistant Acid Phosphatase
FSD: Functional Secretory Domain
IGF: Insulin-like Growth Factor
PDGF: Platelet-Derived Growth Factor
VDR: Vitamin-D Receptor
PTH: Parathyroid Hormone
IL: Interleukin
ER: Estrogen Receptor
CT: Calcitonin
CGRP: Calcitonin Gene-Related Peptide
CTR: Calcitonin Receptor
GPCR: G-protein Coupled Receptor
SERM: Selective Estrogen Receptor Modulator
sCT: Salmon Calcitonin
DMEM: Dulbecco's Modified Eagle Medium
RT: Room Temperature
FBS: Fetale Bovine Serum
mL: Millilitre
HBSS: Hank's Buffered Salt Solution
MQ: Milli-Q
RT-PCR: Reverse Transcription Polymerase Chain Reaction
HRPO: Horseradish Peroxidase
TMB: Trimethylbenzidine
PBS: Phosphate Buffered Saline
ST: Streptavidin
NCON: Negative Control
ELISA: Enzyme-linked Immunosorbent Assay

CHAPTER 1

LITERATURE REVIEW

1.1 NATURE OF BONE

Bone is a living, metabolically active tissue that makes up the human skeleton. It has several key functions in the human body in addition to providing physical support. Bone also acts as a mineral reservoir and serves as a protective environment for bone marrow (Graham *et. al.*, 2006). The strength of bone is determined by its structure as well as its material composition (Currey, 2002). In order to resist deformation, bone needs to be stiff so as to allow for loading to take place on it. However, it must also be flexible in order to withstand compression and tension by widening and narrowing respectively without actually cracking. If bone is stiff and not flexible, it is brittle and therefore energy imposed on it during loading will be released by structural failure. On the other hand, if bone is too flexible and not stiff enough, it will deform beyond its capability and eventually result in cracking. In addition to stiffness and flexibility, bone also needs to be light in order to allow for easy movement. Bone is unique in that it fulfills all of these seemingly contradictory features (Wainwright et al., 1982).

1.1.1 Composition of Bone

Bone tissue is composed of type 1 collagen that is hardened by calcium hydroxyapatite crystals. As mentioned earlier, an increase in the mineral density will increase the stiffness of bone tissue, but that will come at the expense of flexibility. In the body, depending on the role of bone tissue in a particular area, its mineral composition can vary. For example, auditory ossicles are required to be very stiff in order to transmit sound. Therefore, they are highly mineralized when compared to skeletal bone.

Organic Matrix of Bone - Osteoid

Collagen

As described by Viguet-Carrin *et al.* (2006), bone function can also be influenced by the composition and extent of collagen cross-linking as this determines how well bone can absorb energy. Collagens constitute a family of proteins that are present in the extracellular matrix of connective tissues, and are responsible for the maintenance of structural integrity. Collagen is composed of three polypeptide chains called α–chains that form a triple-helix structure. In animals, there are more than 27 different forms of collagen. Some of these types of collagen (I, II, III, V, and XI) are arranged in fibrils, and are found in tissues that have to be able to resist structural stresses like tensile, shear and compression forces. Tendons, bone, cartilage, and skin are some tissues that contain these collagen types. Of these, type I collagen is the most abundant collagen in the body. It can be found extensively in almost all connective tissues, except for hyaline cartilage. Type I collagen is the major protein in bone, skin, tendon, ligaments, cornea, and blood vessels. It comprises about 95% of all collagen in bone, and about 80% of the total proteins in bone (Niyibizi *et al.*, 1994).

Non-collagenous Proteins

As described in 'Anatomy and Physiology of Bone', the organic matrix also contains a number of non-collagenous proteins such as osteocalcin, osteonectin, proteoglycans, growth factors, and serum-derived proteins, among others. Osteocalcin is the best characterized of these proteins, and is thought to be chemotactic for a family of bone cells known as osteoclasts. Osteocalcin is unique to the bone and composes about 15% of the non-collagenous proteins.

Osteonectin is another well-characterized protein, and although it is not unique to bone, it too composes about 15% of the non-collagenous bone protein content. Osteonectin binds strongly function is thought to be in the initiation or moderation of mineralization. Proteoglycans are very abundant in the cartilage matrix, but are also present in the bone matrix. Another group of proteins present in the bone matrix are the growth factors, which comprise insulin-like growth factor (IGF), TGF–β, fibroblast growth factor (FGF), and several other bone morphogenic proteins (BMPs). Some of these proteins are secreted by osteoblasts, which are the bone forming cells. Serum-derived proteins like albumin and immunoglobulin G (IgG) compose about 25% of the non-collagenous proteins in the bone.

Mineralization of Osteoid

The mineral content of bone is dominated by crystalline calcium hydroxyapatite (Ca_{10} $[PO_4]_6$ $[OH]_2$). In addition to this crystalline calcium phosphate salt, there is also some amorphous calcium present in bone, and this is probably the source of exchangeable calcium when rapid adjustments are needed in the extracellular fluid. In addition to calcium and phosphate, bone can also adsorb sodium, magnesium, potassium, citrate, and carbonate ions. Also, ions like fluoride can replace the OH in calcium hydroxyapatite, resulting in the crystal becoming harder and less soluble. The calcium in the crystal can also be replaced by other heavy metals like strontium, plutonium, lead, uranium, and gold.

1.1.2 Structure of Bone

Bone is a unique tissue that is woven at submicroscopic, microscopic, and macroscopic levels in order to be of optimal size, shape, and resistance to

cracking (Yeni *et al.*, 1997). Cortical bone, or compact bone, consists of overlapping functional units called osteons. Osteons are the basic structures of cortical bone, and are the remnants of a resorptive activity. Osteons are formed when osteoclasts and osteoblasts, operating as what Frost (1986) described as a Basic Multi-cellular Unit (BMU), burrow through bone in a direction that is aligned with the long axis of the bone. As described by Parfitt (1994), the osteoclasts form a 'cutting cone' at the front of the BMU, with the osteoblasts following behind them. As the BMU moves through bone, a canal is created and subsequently filled in with connective tissue, blood vessels, and nerves. In other words, the end result of each new BMU is the formation of one new osteon or Haversian system. At the boundary between the cylinder of new bone and the surrounding old bone is a thin layer of cement substance that appears as a cement line when viewed in cross-section. In cortical bone, osteons are parallel to one another much like a wall made up of overlapping bricks (Seeman *et al.*, 2006). The presence of a large number of osteons per unit volume of bone helps limit the propagation of cracks. This is because osteons obstruct the passage of cracks by forcing them to navigate through a high number of osteons. The entry of cracks into an osteon is blocked by the cement line that delimits each osteon, and by the concentric lamellae of mineralized collagen fibers that are packed in various directions. Also, as described by Nalla *et al.* (2004), any uncracked bone tissue that lies within a crack forms a bridge that carries the load that would otherwise propagate the crack forward. Therefore, as described by Qui *et al.* (2005), cracks are largely confined to the older and more densely mineralized bone that lies in between osteons. While the formation of micro-cracks is undesirable, they are usually formed as a last ditch effort to dissipate energy. The alternative to the formation of these micro-cracks is the release of energy via a fracture (Currey, 2002). The

repair of these cracks is carried out by a cycle of bone resorption followed by bone formation, known simply as bone remodeling.

1.1.3 Bone Modeling and Remodeling

As described by Currey (2002), the material composition of bone is crucial towards determining what loads it can tolerate. Conversely, the loads on bone determine its structure and material composition. The adaptation of the material composition and structure of bone to the prevailing loads is carried out by the cellular machinery of bone modeling and remodeling (Parfitt, 1996). In other words, bone modeling and remodeling serve to strengthen the bone to enable load bearing. As the terms suggest, bone formation deposits bone where it is needed while bone resorption removes bone from where it is not needed. During bone modeling, formation and resorption can occur independent of one another – formation alone can take place in order to reinforce a certain area for better load bearing, while resorption alone can take place alone in order to excavate a bone marrow cavity on the inside of bone during growth. Bone remodeling, on the other hand, does not result in a net gain or loss of bone. In bone remodeling, the volume of bone that is resorbed is replaced by an equal volume of new bone. At any moment in time, remodeling may be taking place at focally discrete points on the endocortical, intracortical, and trabecular surfaces all at the same time, with each location in a different stage of remodeling. This, according to Martin and Seeman (2008), is evidence of local regulation of the remodeling cycle. It is also important to note that the resorption phase lasts 2-3 weeks, while the formation phase lasts 2-3 months. Adaptation of the structure of bone to loading is essentially done to minimize stress and avoid damage – especially during growth. However, when damage inevitably occurs within the

mineralized matrix due to fatigue loading or injury, the process of locating, recognizing, and initiating the repair of any damage in the bone is the responsibility of osteocytes. Osteocytes are osteoblasts that have become embedded in lacunae of the bone matrix that they have synthesized. Following entrapment in the bone matrix (also known as osteoid), these osteoblasts undergo a morphological change and become osteocytes with cytoplasmic processes (canaliculi) that link them to lining cells as well as other osteocytes (Han *et al.*, 2004). Upon the inevitable formation of micro-cracks, it is believed that signals are sent out from the site of damage via the damaged matrix and damaged osteocytic processes to the osteoblasts that are either found lining the bone surface adjacent to marrow (in the case of cortical and trabecular bone) or adjacent to a haversian canal on the intracortical surface. The result is the formation of a bone-remodeling compartment (BRC) beneath the flattened osteoblast lining cells near the damage so that osteoclast and osteoblast precursor cells can be recruited to the site (Martin and Seeman, 2008).

When the very fine balance between bone resorption and bone formation is disturbed, it can lead to degenerative bone diseases like osteoporosis.

1.2 OSTEOPOROSIS

Osteoporosis (OP) is a disease that is characterized by the loss of bone mass and deterioration of bone tissue, resulting in bone fragility and increased susceptibility to fractures. In Canada alone, approximately 2 million people suffer from osteoporosis (Osteoporosis Canada, 2009). In the United States, this number of patients suffering from osteoporosis sits at approximately 8 million, and a further 34 million over the age of 50 suffer from low bone mass (osteopenia) that leaves them at risk of developing osteoporosis (US Dept. of

Health and Human Services, 2004). In addition to affecting the quality of life, osteoporosis results in healthcare costs in the region of CAD 1.3 billion per year in Canada (Osteoporosis Canada, 2009) and USD 17 billion per year in the US (Burge et al., 2007). Numerous studies have shown that OP does not affect everyone to the same degree. For example, the National Osteoporosis Foundation in 2002 reported that of the estimated 44 million Americans suffering from low bone mass, 80% are women.

1.2.1 Diagnosis

Osteoporosis can be diagnosed clinically, radiographically, or by measurements of bone density. Clinically, the disease can present itself with low-impact fractures, or fragility fractures (Mauck and Clarke, 2006), but OP is most commonly diagnosed by measuring bone density using Dual Energy X-ray Absorptiometry (DEXA). DEXA works by passing a beam of x-ray photons through the bone region of interest to determine its density (Inzerillo and Zaidi, 2002), and an osteoporosis diagnosis can be made when a DEXA scan of the total hip, femoral neck, or lumbar spin has a T-score of -2.5 or less (U.S. Preventative Task Force, 2002; World Health Organization, 2003). This criterion, as described by Riggs and Wahmer (1988), identifies approximately 30% of postmenopausal women as having OP. An interesting alternative to DEXA is the use of quantitative computed tomography. qCT is used to assess bone mineral density (BMD), and according to Inzerilla and Zaidi (2002), is the only method that allows for volumetric bone density measurement. However, qCT is very expensive and is limited by radiation exposure. DEXA, as stated by Kanis *et al.* (1994) is preferred over qCT in terms of accuracy, precision, radiation exposure and quality control in a clinical setting (Kanis *et al.*, 1994). The advances in bone

density measurement include techniques such as lateral vertebral assessment (LVA) using DEXA, as well as techniques like magnetic resonance imaging (MRI) and microcomputed tomography (μCT). These newer techniques measure actual bone morphology as a measure of bone 'quality' in contrast to DEXA, which only provides quantitative information about the bone density (Inzerillo and Zaidi, 2002). Biochemical markers of bone turnover in serum or urine are currently not recommended for diagnosis of osteoporosis (World Health Organization, 2003; Agency for Healthcare Research and Quality, 2001). The US Preventative Task Force in 2002 recommended bone density testing for all women over the age of 65 years, and for women between 60 and 64 that are at high risk of OP. For men, the USPTF reccomends bone density testing those with the following conditions: history of non-traumatic fracture, radiographic evidence of osteopenia, chronic glucocorticoid use, hypogonadism, and hyperthyroidism. In 2003 (The Writing Group for ISCD Position Development Conference) and 2005 (Binkley *et al.*), the International Society for Clinical Densitometry (ISCD) reccomends bone density testing for all men over the age of 70.

1.2.2 Cause and Progression

The peak bone density in both men and women is achieved in the early part of life - usually in the early 20s (Campion and Maricic, 2003). Studies have shown that even though the peak densities in men and women are very similar, men have 10-12% greater peak bone mass and greater bone size. Campion and Maricic (2003) state that the bone loss usually begins when men and women are about 50 years of age, and this is due to an increase in osteoclast-mediated bone resorption, increased cortical porosity, and endocortical thinning. This excess bone loss is not nullified by increased bone formation, and as a result

there is a net loss of bone. The reason for higher rates of OP in women is because the changes in bone coincide with the onset of menopause and estrogen deficiency. Estrogen-deficient bone loss initially occurs at a much higher rate than other age-related factors involved in bone loss, and so bone loss and the onset of OP is accelerated among post-menopausal women in comparison to men. A study by Sigurdsson *et al.* (2006) found that when they compared the extent of bone loss in men and women of the same age group, the women had a 2 to 5 times greater loss of bone mass resulting in a 2-fold decrease in bone strength when compared to men of the same age. In both men and women, the two most important independent risk factors for initial vertebral and non-vertebral fractures are age and low BMD (van der Klift *et al.*, 2005). Low body mass index (BMI) and body weight are associated with low BMD and increased fracture risk in post-menopausal women as well as in men. According to van der Klift *et al.* (2005) BMI has been shown to be a good surrogate marker for BMD and may in fact be a good indicator of low bone mass. Studies by Lim *et al.* (2004) have found that age-related changes in body composition also have gender-specific impacts on BMD. For instance, the age, weight, BMI, lean body mass (LBM) and fat mass (FM) in men was significantly correlated with their BMD. In women, weight, BMI, LBM, FM, years since menopause, number of deliveries, and number of children breastfed all significantly correlated with BMD. Goderie-Plomp *et al.* (2004) found that low serum estradiol levels in post-menopausal women was associated with both low BMD and with an increased risk of vertebral fracture that is independent of BMD. Men with low testosterone were not found to correlate with low BMD or incidence of vertebral fracture (Campion and Maricic, 2003; Goderie-Plomp *et al.*, 2004). The World Health

Organization in 2003 suggested that low estradiol levels in men correlated more strongly to a decrease in BMD than low testosterone levels in women.

1.3 BONE RESORPTION

As described by Martin and Seeman (2008), the sequential functions involved in bone remodeling are most likely regulated by cellular and molecular events that are multidirectional. Remodeling is very tightly regulated by systemic, central, and local factors that provide signals to and within the multicellular unit that is comprised primarily of osteoclasts and osteoblasts. The other cells involved in the multicellular unit are osteocytes and immune cells. As mentioned earlier, micro-crack formation results in the damaging of osteocytic processes in their canaliculi. This, according to Hazenberg *et al.* (2006), results in the apoptosis of osteocytes, eventually resulting in the formation of a bone-remodeling compartment (BRC). As described by Taylor (1997) and Verbogt *et al.* (2000), the number of apoptosed osteocytes can provide the topographical information needed to identify the location and size of damage. Therefore, osteocytes apoptosis is very likely to be one of the first events signaling the need for remodeling, and even precedes osteoclastogenesis (Clark, *et al.*, 2005). *In vivo*, osteocyte apoptosis takes places within 3 days of immobilization and is followed by osteoclastogenesis within 2 weeks (Aguirre, *et al.*, 2006).

Osteoblast Recruitment

While bone resorption by osteoclasts precedes bone formation by osteoblasts, the recruitment of osteoblasts progenitors may in fact precede the recruitment of osteoclasts progenitors. The reason for this is that osteoblasts have a membrane bound protein, Receptor activator of NFκB Ligand (RANKL),

that is required by osteoclast precursors to differentiate into bone resorbing cells (Chambers, 1982). Therefore, osteoblasts must be present at the site of resorption in order for osteoclasts to form and subsequently resorb bone. Osteoblasts may arise from one of several sources. They can arise from mesenchymal precursors in bone marrow, from mesenchymal cells in the endosteal canopy of the bone-remodeling compartment (BRC), or from circulation (Hauge *et al.*, 2001; Parfitt 2001).

Osteoclast Formation and Recruitment

As described by Väänänen and Laitala-Leinonen (2008), it is now well established that osteoclasts are members of the monocytic macrophage lineage and are formed via cellular fusions from their mononuclear precursors. This was first described by Walker (1973) when they found that circulating blood in parabiotic animals contained cells that were capable of forming resorbing osteoclasts in osteopetrotic (excess bone formation) animals. Later *in vitro* studies such as those conducted by Burger *et al.* in 1982 suggested that osteoclasts were derived from macrophage precursors of colony-forming unit-macrophage (CFU-M) lineage. Osteoclasts therefore share a common hematopoietic origin with antigen-presenting dendritic cells and other tissue macrophages such as alveolar macrophages. Väänänen and Laitala-Leinonen (2008) also pointed out in their review that it is not yet known whether all circulating monocytes are capable of differentiating towards the osteoclast lineage, or if it is a unique feature of a subset of monocytes. It is known, however, that all monocytes can be triggered to differentiate towards the osteoclast lineage by the ligand of receptor activator of nuclear factor-KB (RANKL) and macrophage-colony stimulating factor (MCSF). The details

regarding the triggering of osteoclast formation by RANKL and MCSF will be discussed in detail in the next section.

1.3.1 OSTEOCLAST BIOLOGY

1.3.1.1 Triggering of Osteoclastogenesis

As established by Lacey *et al.* (1998) and Yoshida *et al.* (1998), two cytokines that are essential and sufficient to induce osteoclastogenesis and to regulate osteoclast activity are the macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor κB ligand (RANKL). M-CSF and RANKL are both secreted by/present on bone marrow stromal cells and osteoblasts (Destaing *et al.*, 2003). M-CSF induces the expression of RANKL's receptor, Receptor activator of NFκB (RANK), in osteoclast precursors thus priming these cells to differentiate into osteoclasts in the presence of RANKL. The deletion of either one of M-CSF, RANK, or RANKL results in the inhibition of osteoclastogenesis thus leading to osteopetrosis in mice (Suda *et al.*, 1992; Teitelbaum, 2000). We will now discuss in detail the roles that M-CSF, RANK, and RANKL play in osteoclastogenesis.

1.3.1.1.1 Macrophage Colony Stimulating Factor (M-CSF)

Macrophage Colony Stimulating Factor, abbreviated as M-CSF or CSF-1, is found at biologically active concentrations in the circulation and regulates the survival, proliferation, and differentiation of mononuclear phagocytes from undifferentiated precursors into fully differentiated macrophages. The first indication of the involvement of M-CSF in bone physiology came in 1990, when

Wiktor-Jedrzecjak et al. and Yoshida et al found that osteopetrotic mice (*op/op*) were deficient in M-CSF and lacked functional osteoclasts. This suggested that M-CSF played a critical role in the formation of osteoclasts, and therefore had a major influence on bone resorption. Felix *et al.* (1990) then demonstrated a 15-day regimen of daily M-CSF injections reversed the osteopetrotic phenotype. Dai *et al.* (2002) further confirmed that M-CSF deficiency was the cause of the osteopetrosis when they were able to replicate the phenotype by knocking out the M-CSF receptor c-fms.

M-CSF / c-fms Signaling Pathway

Activation of c-fms by M-CSF promotes the proliferation and survival of cells of the monocytic lineage, including osteoclasts and osteoclast precursors. In addition, M-CSF is essential for the expression of the receptor activator of NFκB (RANK), which upon binding to its ligand RANKL, results in osteoclastogenesis (Arai *et al.*, 1999). M-CSF signaling also induces cytoskeletal reorganization, cell spreading, and increased motility of mature osteoclasts (Fuller *et al.*, 1993; Insogna *et al.*, 1997a; Insogna *et al.*, 1997b). The autophosphorylation of tyrosine residues on c-fms following its interaction with M-CSF results in the creation of binding sites for several phosphotyrosine-binding proteins such as the Src family kinases (SFK), c-Cbl, phosphoinositide 3-kinase (PI3K), and Grb2 (Faccio *et al.*, 2007). The role of the SFKs like c-src in osteoclasts has been proposed to be in the formation of membrane ruffles and cell motility (Insogna *et al.*, 1997a; Faccio *et al.*, 2003). Faccio et al. confirmed this in 2007 when they found that the deletion of the tyrosine residue responsible for SFKs binding resulted in the complete loss of these features. In contrast to other studies, Faccio *et al* (2007) found that direct binding of PI3K to its tyrosine phosphate

residues on c-fms is not essential for signaling to the cytoskeleton. Another protein, c-Cbl, is known to be a regulator of podosome assembly/disassembly and membrane ruffling in macrophages, and for migration of macrophages as well as osteoclasts (Caveggio *et al.*, 2003; Chiusaroli *et al.*, 2003).

Faccio *et al.* (2007) suggested that PI3K could be in fact be indirectly recruited to a signaling complex formed at the SFK-binding Tyr-559 residue on c-fms. The authors also found that SFKs do not associate directly with PI3K because the deletion of c-Cbl resulted in there being no interaction between SFK and PI3K. Therefore, they proposed a scenario wherein first there is an interaction between SFKs and c-Cbl, and then c-Cbl and PI3K, eventually resulting in the formation of a SFK-c-Cbl-PI3K complex. Faccio *et al.* (2007) went on to propose that for successful cytoskeletal signaling by this complex requires the small GTPase Rac, which is activated by the phosphorylated guanosine exchange factor Vav3, which in turn is activated by M-CSF occupation (Faccio *et al.*, 2003). As described by Meng and Lowell in 1998, many osteoclast-regulating pathways including integrin signaling and NFκB translocation are in downstream reactions mediated or modulated by c-src and c-Cbl. This interaction between M-CSF and integrins are described below.

M-CSF and Integrins

Integrins are molecules that mediate cell/matrix recognition, and are generally α/β heterodimers that consist of long extracellular and relatively short intracellular domains. These domains function to attach cells to extracellular matrices, and also help transmit matrix-derived signals to the cells. McHugh *et al.* (2000) found that the αv family of integrins is differentially expressed by

osteoclasts during their maturation. They also found that two members, namely αvβ3 and αvβ5, are functional in these cells. In marrow macrophages that have been exposed to just M-CSF, the predominant integrin seen is αvβ5, and not αvβ3 (McHugh *et al.*, 2000). Upon exposure to RANKL, the αvβ5 disappears to be replaced by αvβ3 (Inoue *et al.*, 1998). The importance of αvβ3 integrin was demonstrated by studies that showed that while αvβ5 deficiency accelerated bone loss in estrogenopryvic animals (Lane *et al.*, 2005), oophorectomized animals that lacked αvβ3 were actually protected from bone loss (Zhao *et al.*, 2005). The αv family of integrins recognizes the amino acid motif Arg-Gly-Asp (RGD), which is present on a number of bone matrix proteins such as osteopontin and bone sialoprotein. The presence of these ligands activates the integrin by changing its conformation (Takagi *et al.*, 2002) by a process known as outside-in signaling. This form of signaling induces a number of intracellular events, with one of the most important ones being the organization of the actin cytoskeleton. αvβ3 integrin is also modulated by an inside-out mechanism that is stimulated by intracellular events such as M-CSF binding its receptor, c-fms (Faccio *et al.*, 2003). The collaborative relationship that αvβ3 and c-fms share is illustrated by the capacity of a high dose of M-CSF to rescue retarded osteoclastogenesis that occurs upon deleting a β3 integrin subunit (Faccio *et al.*, 2003). As described by the authors, although the exposure of αvβ3-deficient osteoclasts to high-doses of M-CSF rescues osteoclastogenesis and cytoskeletal organization, the main role of αvβ3 integrin is to aid the cell in the resorption of bone. Engelman *et al.* (1997) found that αvβ3 inhibitors (competitive ligands) in fact arrest bone resorption, suggesting that αvβ3 is the principal integrin expressed by osteoclasts. In order to study this further, McHugh *et al.* (2000)

deleted the β3 integrin subunit in mice. They found that mice lacking αvβ3 integrin generated osteoclasts that were incapable of optimal resorptive activity as their ruffled membranes and actin rings were abnormal *in vivo*. Studies by Faccio *et al.* (2003) have also shown that M-CSF and αvβ3 collaboratively induce cytoskeletal organization by converting Rho family proteins, RhoA and Rac, from their inactive GDP-bound state to their active GTP-bound states. According to the authors, this observation suggests that molecules that regulate Rho family GTPases may in fact mediate integrin activation. As described by Faccio *et al.* (2005), a Rac-specific guanine nucleotide exchange factor (GEF) called Vav3 is essential for organizing the cell's cytoskeleton and therefore for its resorptive activity. Consequently, the authors found that Vav3-deficient osteoclasts fail to activate Rac in response to M-CSF or αvβ3 occupancy.

M-CSF Interaction with Integrins and ITAMs

It has been shown that αvβ3, upon activation, recruits the tyrosine kinase Syk to their cytoplasmic domains, where it is phosphorylated by c-src. Syk is in turn is a crucial upstream regulator of Vav3. These events, as described by Koga *et al.* (2004), occur in the context of ITAM (immunoreceptor tyrosine-based activation motif) proteins DAP12 (DNAX-activating protein 12) and FcTγ (Fc receptor common γ subunit), which when deleted result in the arrest of terminal osteoclastogenesis due to failed expression of the crucial transcription factor NFATc1.

As described by Faccio *et al.* (2003) as well as Teitelbaum and Ross (2005), c-fms and integrin αvβ3 both organize the osteoclast cytoskeleton in a collaborative manner that involves the sharing of some effector molecules that

are common to both. Therefore, Zou *et al.* (2008) conducted studies to see if M-CSF, like integrin $\alpha v\beta 3$, mediated cytoskeletal organization via DAP12. Similar to integrin occupancy, M-CSF also regulates the cytoskeleton by using SFKs like c-Src, which phosphorylate ITAM motifs in other cell types. They also made sure to use $\alpha v\beta 3$ – deficient osteoclasts in order to prove that $\alpha v\beta 3$ did not couple c-fms to DAP12 in any way. The results showed that c-fms activation did in fact phosphorylate DAP12 in a c-src-dependent manner, and also resulted in the downstream phosphorylation of Syk. Syk then mediates the cytoskeletal organization and actin-ring formation that occurs following M-CSF binding to its receptor, resulting in bone resorption (Zou *et al.*, 2008).

M-CSF and Osteoclast Survival

M-CSF also plays a role in regulating osteoclast survival by preventing apoptosis. Apoptosis is a genetically programmed form of cell death that can be triggered by a number of pathological and physiological stimuli. M-CSF signaling triggers PI3K and the anti-apoptotic Akt kinase in osteoclasts. Akt activity, as described by Datta *et al.* (1999) and Kelley *et al.* (1999), is necessary for cell survival. Akt has been shown to target the apoptotic machinery by phosphorylating downstream pro-apoptotic molecules such as BAD, caspase-9, glycogen-synthase kinase and forkhead family members, thus preventing apoptosis of osteoclasts. Another interesting layer in the control of osteoclast apoptosis involves the mammalian target of rapamycin (mTOR), which is a serine/threonine kinase that plays a crucial role in the control of protein translation. mTOR and p70 ribosomal protein S6 kinase (S6K) are regulated via PI3K effectors, including Akt and phosphoinositide-dependent kinase 1 (PDK-1)

(Inoki *et al.*, 2002; Potter, C.J. *et al.*, 2002; Tee *et al.*, 2002; Alessi *et al.*, 1998). Studies conducted by Glantschnig *et al.* in 2003 found that the anti-apoptotic pathway involving mTOR/S6K is commonly engaged by M-CSF, TNFα and RANKL, and noted that inhibition of mTOR resulted in osteoclast apoptosis and suppression of bone resorption *in vitro*, suggesting that mTOR control of protein translation can be rate limiting in the control of an osteoclast's lifespan.

1.3.1.1.2 Receptor Activator of Nuclear Factor κB (RANK)

The receptor activator of nuclear factor κB (RANK) belongs to the tumor necrosis factor receptor (TNFR) superfamily and is expressed on osteoclasts and their precursors, hematopoietic precursors, dendritic cells, and mammary epithelial precursors. RANK is also known as TNFRSF11a, OFE, ODFR, TRANCE-R, ODAR, and CD265, and was first cloned from a bone marrow derived myeloid dendritic cell (DC) cDNA library enhancing DC survival (Anderson *et al.*, 1997). RANK cDNA in humans and mice encodes type I transmembrane glycoproteins consisting of 616 (~80kDa) and 625 (~60kDa) amino acids respectively. In humans, the transmembrane glycoprotein consists of 29 amino acid signal peptides while the mouse transmembrane glycoprotein is comprised of 30 amino acids. Human and mouse RANK cDNA comprises an extracellular domain of 183 and 185 amino acids respecitively, as well as a transmembrane domain of 21 and 20 amino acids and a large cytoplasmic membrane of 383 and 391 amino acids respectively. TNFR superfamily receptors such as FAS, TNFR1 and TNFR2 all generally assemble into trimeric complexes on the cell surface prior to ligand binding, and so it has been suggested that trimerization of RANK is a prerequisite for the binding and signal transmission of

its ligand (RANKL) (Chan *et al.*, 2000; Siegel *et al.*, 2000; Chan *et al.*, 2000; Locksley *et al.*, 2001). RANK can be detected on the surface of dendritic cells (Anderson *et al.*, 1997; Wong *et al.*, 1997; Williamson *et al.*, 2002), CD4+ and CD8+ T-cells (Josien *et al.*, 1999), and on mammary epithelial cells where expression is regulated throughout the course of pregnancy (Fata *et al.*, 2000).

1.3.1.1.3 Receptor Activator of Nuclear Factor κB Ligand (RANKL)

Receptor activator of nuclear factor κB ligand (RANKL) is a member of the TNF family, and is the ligand for RANK. RANKL was cloned based on its strong up-regulation in T-cells following antigen receptor stimulation and was shown to supplement the ability of dendritic cells to stimulate naïve T-cell proliferation (Anderson *et al.*, 1997; Wong *et al.*, 1997). As described by Leibbrandt and Penninger (2008), the RANKL gene codes for a type II transmembrane protein that contains a membrane-anchoring domain, a connecting stalk, and a receptor-binding ectodomain. Human and mouse RANKL consist of 317 and 316 amino acids respectively, and are very similar. As is the case with all other members of the TNF family of cytokines, the crystal structure of the extracellular biologically active domain of murine RANKL revealed that the protein self-assembled itself into non-covalently associated trimers (Lam *et al.*, 2001; Ito *et al.*, 2002). RANKL mRNA shows high levels of expression in skeletal tissues, primary and secondary lymphoid tissues, keratinocytes of the skin, mammary gland epithelial cells, the heart, skeletal muscle, the lungs, the stomach, placenta, the thyroid gland, and even the brain (Anderson *et al.*, 1997; Lacey *et al.*, 1998; Yasuda *et al.*, 1998; Wong *et al.*, 1997; Kartsogiannis *et al.*, 1999; Fata *et al.*, 2000; Loser *et al.*, 2006). In addition to the ~40 kDa membrane-bound form, RANKL can also exist as a soluble ~31 kDa protein that is derived either through proteolytic

cleavage or through alternative splicing. The analysis of the RANKL gene by Ikeda *et al.* (2003) revealed the presence of two alternative transcripts. One encoded a membrane-bound isoform comprising of 287 amino acids with a short intracellular domain while the other coded for a soluble protein made up of 199 amino acids and lacking both the transmembrane and intracellular domains of the canonical RANKL. Studies into the efficacy of membrane-bound RANKL versus soluble RANKL when it comes to osteoclastogenesis have yielded contradictory results. One study found that murine models of cancer showed increased MMP-7 expression by osteoclasts at the tumor-bone surface, and this leads to the conversion of membrane-bound RANKL into soluble RANKL thereby promoting osteoclast activation and subsequently bone resorption (Lynch *et al.*, 2005). Meanwhile, another study by Hikita *et al.* (2006) suggests that membrane-bound RANKL induces osteoclastogenesis more efficiently than soluble RANKL. They also suggested that MMP-14 mediates the ectodomain shedding of RANKL, and that this negatively affects osteoclastogenesis. They supported this observation by the osteoporotic phenotype of MMP-14-deficient mice, a condition that the authors attribute to the increased osteoclast numbers due to the increase in membrane-bound RANKL. To echo the thoughts of Leibbrandt and Penninger (2008), the effect of membrane-bound and soluble RANKL on osteoclastogenesis needs to be studied in more detail in order to gain a better understanding of the subject.

1.3.1.1.4 Osteoprotegerin (OPG)

Osteoprotegerin (OPG) was the first to be discovered in the trio of RANKL, RANK, and OPG. The discovery was made by two different groups, almost simultaneously, based on its ability to inhibit osteoclast development *in vitro* and

in vivo (Tsuda *et al.*, 1997; Simonet *et al.*, 1997). OPG, like RANK, is a member of the TNF receptor superfamily. However, unlike the other members of this family of receptors, it lacks a transmembrane domain. This suggests that OPG is secreted from the cell as a soluble protein. Human and mouse OPG are about 85% identical, and it is a glycoprotein that is first synthesized as a protein precursor consisting of 301 amino acids before it is processed to the mature form which consists of 280 amino acids. The loss of amino acids is as a result of the cleaving of the 21-amino acid signal peptide. The monomeric form of OPG (~55kDa) is converted to the predominant extracellular form of OPG is that of a disulfide-linked homodimer (~110 kDa) that is found in the brain, liver, lungs, heart, kidneys, skeletal muscle, skin, intestines, calvaria, stomach, testes, and placenta (Yasuda *et al.*, 1998; Tsuda *et al.*, 1997; Simonet *et al.*, 1997; Tan *et al.*, 1997; Kwon *et al.*, 1998).

The RANKL/RANK/OPG Axis

The first molecule identified in the RANKL-RANK-OPG axis was OPG, and it was initially cloned as it was seen to be a potential inhibitor of osteoclastogenesis (Tsuda *et al.*, 1997; Simonet *et al.*, 1997). Studies found that transgenic mice that over-expressed OPG and mice that were treated with OPG both showed increases in their bone density (Simonet *et al.*, 1997). Subsequent studies found that the targeted deletion of OPG in mice resulted in early-onset osteoporosis. In addition to cementing the idea that OPG was a critical component in the maintenance of bone strength, these studies also suggested that OPG might be accomplishing this by neutralizing a TNF-related factor that stimulated osteoclast development (Leibbrandt and Penninger, 2008). Subsequent studies found that RANKL was a stimulator of osteoclastogenesis

and that it binds specifically to OPG, resulting in the inhibition of RANKL's ability to promote osteoclastogenesis. Hence, RANKL was identified as a novel factor involved in osteoclast development and function. Evidence from a landmark study found that the targeted deletion of RANKL in mice resulted in their development of osteopetrosis (Kong *et al.*, 1999). Similar results were seen when the receptor RANK was deleted in mice (Dougall *et al.*, 1999; Li *et al.*, 2000), hence establishing the crucial role of the RANKL-RANK interaction in the enhancement of osteoclastogenesis and the role of the decoy receptor OPG as a counter-balance. As described by Leibbrandt and Penninger (2008) essentially all factors that inhibit of enhance bone resorption by osteoclasts also influence the levels of RANKL and OPG mRNA or proteins. As a result, the authors have concluded that the RANKL-RANK-OPG axis is at the heart of the process of osteoclast-mediated bone remodeling.

Since these landmark discoveries, the RANKL-RANK-OPG axis has been implicated in several human bone diseases. For instance, studies have found that patients suffering from the metabolic bone disease expansile skeletal hyperphosphatasia have an insertion in exon 1 of RANK, a mutation that has been mapped as the cause of the disease (Whyte *et al.*, 2000; Whyte and Hughes, 2002). Also, mutations affecting the ligand-binding domain of OPG have been found in patients suffering from juvenile Paget disease, which is an autosomal recessive osteopathy characterized by osteopenia (Whyte and Hughes, 2002; Cundy *et al.*, 2002; Chong *et al.*, 2003).

RANKL-RANK Signaling Pathway

When discussing the RANKL-RANK signaling pathway, it is important to keep in mind the fact that the expression patterns of both the ligand and the receptor are broad and as a result their functions are pleiotropic in nature. As a result, there is a lot of crosstalk with other receptor/ligand systems in different cells. Members of the TNF-receptor superfamily, such as RANK, do not possess any kinase activity. As a result, they recruit associated factors in order to achieve signal transduction and these factors are known as TNFR-associated factors (TRAFs). As described by Inoue et al. (2000), TRAFs mediate ligand-induced signal transduction by binding various regions within the cytoplasmic tail of TNF receptors. The cytoplasmic tail of RANK contains several TRAF binding sites that cluster into several distinct domains. For example, the region spanning amino acids 235-358 and amino acids 359-531 binds TRAF 6 and the region containing amino acids 532-625 contain multiple binding sites for TRAFs 2, 5, and 6 (Darnay *et al.*, 1998; Wong *et al.*, 1998; Wong *et al.*, 1999). These TRAF binding domains mediate RANK-induced NFκB and c-Jun NH2-terminal kinase (JNK) activation. The deletion of the membrane-proximal TRAF6 binding domain results in the inhibition of RANK-mediated NFκB signaling, but JNK activation remains unaffected. This suggests that TRAF6 is essential for NFκB activation but not for JNK activation (Darnay *et al.*, 1998; Wong *et al.*, 1998; Galibert *et al.*, 1998; Lee *et al.*, 2000). The importance of TRAF6 as a downstream transducer of RANK signaling was demonstrated by studies wherein Traf6 gene-deficient mice were observed to exhibit phenotypes similar to $RANKL^{-/-}$ and $RANK^{-/-}$ mice as a result of the partial blockage of osteoclastogenesis and defective activation of mature osteoclasts (Lomaga *et al.*, 1999; Naito *et al.*, 1999; Kobayashi *et al.*, 2001;

Kobayashi *et al.*, 2003). In comparison to TRAF6, TRAF2 and TRAF5 play a very minor role in osteoclastogenesis. In addition to the TRAFs, another adaptor protein has been identified as being an important part of RANK/RANKL signaling. Grb2 (or Gab2) has been found to associate with RANK and mediate RANK-induced NFκB, Akt, and JNK activation. Mice lacking Grb2 display an osteopetrotic phenotype and decreased bone resorption due to defective osteoclast differentiation (Wada *et al.*, 2005).

As described above, RANKL-RANK signaling involves the activation of the transcription factor NFκB. Leibbrandt and Penninger (2008) stated in their publication that mammals express five NFκB proteins (also known as Rel proteins) that belong to two classes. The first class consists of the proteins Rel A (p65), c-Rel, and RelB. All of these are synthesized as mature products and do not require any processing. Proteins of the second class, NFκB-1and NFκB-2, are first synthesized as precursors p105 and p100 before being processed proteolytically into mature p50 and p52 NFκB proteins respectively. For osteoclast development, the expression of both NFκB proteins (p50 and p52) is required. In 1997, Franzoso *et al.* and Iotsova *et al.* found that $p50^{-/-}$ and $p52^{-/-}$ mice develop osteopetrosis due to the lack of osteoclasts and defective osteoclast differentiation, respectively, thus establishing the importance of the two proteins. As described by Karin and Lin (2002) and Hayden and Ghosh (2008), the p50 and p52 proteins are actually tied up in a cytoplasmic complex with RelA or c-Rel through an association with IκB inhibitor proteins. This inhibitor protein degrades upon activation of the pathway by means of a specific phosphorylation by activated IκB kinase (IKK). The IKK complex consists of two

highly homologous catalytic subunits IKKα and IKKβ, and studies by Ruocco *et al.* (2005) have suggested that it is IKKβ that is essential for the activation of the classical NFκB pathway that controls osteoclastogenesis *in vivo*. Leibbrandt and Penninger (2008) also described another signaling pathway that is triggered by the binding of RANKL to RANK: the mitogen-activated protein kinases (MAPK) pathway. MAPKs belong to the Ser/Thr protein kinase family, and consist of extracellular signal-regulated kinases (Erk1/2), p38-MAPKs (α/β/γ/δ), JNKs (JNK1, 2, 3), and larger MAPKs (Erk5, 7, 8). Several MAPKs have been shown to be activated downstream of RANK, and they help integrate RANK activation to a cellular response.

The transcription factor NFATc1 (nuclear factor of activated T-cells 1) was first identified while researchers were looking for factors that were specifically induced by RANKL, but not by the cytokine IL-1, during osteoclastogenesis (Takanayagi *et al.*, 2002). The expression of NFATc1 has been shown to depend on the NFκB and c-Fos pathways, and the ectopic expression of NFATc1 has resulted in osteoclast differentiation *in vitro*. Members of the NFAT family also require the presence of Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase calcineurin for their activation and nuclear translocation (Leibbrandt and Penninger, 2008). Studies have found that both, the chelation of Ca^{2+} ions in the medium as well as the specific inhibition of calcineurin resulted in the blocking of osteoclast differentiation even in the presence of RANKL *in vitro*. This suggests that RANKL-mediated Ca^{2+} levels are crucial for the proper differentiation of osteoclasts; whereby NFATc1 is activated through the Ca^{2+}-dependent calcineurin pathway (Takayanagi *et al.*, 2002; Asagiri *et al.*, 2005; Takayanagi, 2007).

Another important pathway that RANKL-RANK signaling is involved in is the release of Ca^{2+} from intracellular stores by acting through the phosphoinositide-related enzyme PLC (phosphoinositide-specific phospholipase C). PLC is responsible for catalyzing the production of the second messenger inositol (1,4,5) – triphosphate (IP3) and diacylglycerol (DAG) from PI (4,5) P2. The release of Ca^{2+} by RANKL results in the acceleration of the translocation of NFκB to the nucleus, thereby promoting osteoclast survival (Takayanagi *et al.*, 2002). Eventually, the increased levels of Ca^{2+} result in the activation of NFATc1 via calcineurin. Inhibition of PLCγ enzymatic activity has been shown to impair osteoclast development and function, and PLCγ2$^{-/-}$ mice display an osteopetrotic phenotype. As described by Mao *et al.* (2006), PLCγ2 is required for the differential regulation of NFATc1, AP-1, and NFκB. The activation of PLCγ by RANK has been shown to require the nonreceptor tyrosine kinase Syk and ITAM (immunoreceptor tyrosine-based activation motif)-bearing molecules such as DAP12 and FcRγ, and the absence of DAP12 and FcRγ in mice results in a highly osteopetrotic phenotype. Studies by Takayanagi *et al.* (2002), Mao *et al.* (2006), and Takayanagi *et al.* (2007) found that NFATc1 regulation was dependent on RANKL-mediated phosphorylation of PLCγ2 downstream of DAP12/FcRγ, and also that PLCγ2 can associate with the regulatory adaptor molecule Grb2 in osteoclasts and recruit it to RANK.

Besides the calcineurin-NFATc1 pathway, protein kinase C (PKC) proteins are also activated by Ca2+ ions. Studies have linked mutations in the PKC scaffolding protein p62 to Paget's disease. Similar to TRAF6, p62 becomes upregulated during RANKL-induced osteoclastogenesis and the inactivation of

p62 results in impaired osteoclastogenesis as well as the inhibition of IKK activation and NFkB nuclear translocation.

Crosstalk with Other Signaling Pathways

RANKL-RANK signaling is not restricted to osteoclasts and their precursors, but is in fact seen on several other cell types. The result is crosstalk between other signaling molecules, as discussed below.

Mammary glands

Studies have show that RANKL-RANK signaling controls the development of lactating mammary glands in pregnancy, with the highest levels being at day 15.5 in mice (Fata *et al.*, 2000; Gonzalez-Suarez *et al.*, 2007).

Lymph node development

Several studies that looked at transgenic $Rankl^{-/-}$ and $Rank^{-/-}$ mice found that there was a complete absence of lymph nodes (Dougall *et al.*, 1999). This lead to further studies involving patients with an osteoclast-poor form of the autosomal recessive osteoporosis (ARO), where they found the cause of the disease to be various mutations in RANKL (Sobacchi *et al.*, 2007). The same research then found that these patients showed no palpable signs of lymph nodes, further strengthening the theory that RANKL-RANK signaling is involved in lymph node formation in humans.

T-Cells

One important site of RANKL-RANK signaling is in activated T-cells, where RANKL is induced upon antigen-receptor engagement. Studies by Kong *et*

al. (1999) showed that activated CD4+ T-cells were indeed capable of inducing osteoclastogenesis in a hematopoietic bone marrow precursor cell culture system. Studies have since established the major role of activated T-cells in the resorption of bone via the up-regulation of RANKL. For instance, knocking out the cytotoxic T lymphocyte–associated protein 4 (Ctla4) in mice results in the spontaneous activation of T-cells, and the mice display a severe osteoporotic phenotype. This leads to the question: why do T-cells not cause extensive bone loss every time they encounter foreign antigens? The answer is that T-cells have been shown to also secrete a factor that negatively affects RANKL-RANK signaling. This molecule has been identified as IFN-γ, and it has been found to promote the accelerated degradation of TRAF6 thus resulting in the inhibition of RANKL-induced NFκB and JNK activation (Takayanagi *et al.*, 2000). RANKL can also induce IFN-β production in osteoclast precursor cells, and this factor has been shown to inhibit osteoclast differentiation by disrupting the RANKL-mediated expression of c–Fos. As described by Leibbrandt and Penninger (2008), this suggests that an autoregulatory loop exists wherein RANKL-induced c-Fos induces its own inhibitor IFN-β. This suggestion is supported by the fact that IFN-$\beta^{-/-}$ mice exhibit enhanced osteoclastogenesis resulting in osteopenia (Takayanagi *et al.*, 2002).

Dendritic Cells

Dendritic cells (DCs) are cells that are programmed to capture and process antigens in the body. Usually, DCs exist in an immature state that leaves them unable to stimulate T-cells. Upon coming in contact with an antigen, however, the DCs mature and carry the captured antigens to areas of the body

that are rich in T-cells, such as secondary lymphoid organs. In this T-cell rich area, DCs present the antigen to antigen-specific T-cells. The DCs and T-cells then interact with B-cells to initiate adaptive immune responses (Banchereau and Steinman, 1998). As described by Ingulli *et al.* (1997), antigen-bearing DCs are in direct contact with naïve T-cells in the lymph nodes and are eliminated soon as they interact with the T-cells. The apoptosis of DCs is triggered by the activated T-cells that produce TNF-related apoptosis-induced ligand (TRAIL), FasL, and TNF-α. While RANKL is not expressed on resting CD4+ or CD8+ T-cells, surface RANKL is detected on these cells following their antigenic stimulation (Josien *et al.*, 1999).

1.3.1.2 Formation of a multinucleated osteoclast

Cell fusion is one of the most characteristic properties of osteoclasts and is formed through the fusion of homotropic monocyte precursor cells (Ishii and Saeki, 2008). The fusion of osteoclasts has been shown to be very important for efficient bone resorption, but the absence of multinucleation does not completely inhibit resorptive activity (Kukita *et al.*, 2004; Yagi, *et al.*, 2005; Baylies *et al.*, 1998). This is thought to be because the function of multinucleation is primarily to increase the size of osteoclasts so as to enable them to resorb larger regions of bone tisses in a given time (Ishii and Saeki, 2008). As described by Teitelbaum and Ross (2003), this is important as osteoclasts can only resorb the area of bone that is sealed off directly under them. Some of the factors involved in the process of osteoclastogenic cell fusion are discussed below.

MFR/ SIRPα

Saginario *et al.* (1995) and Saginario *et al.* (1998) identified the macrophage fusion receptor (MFR), also known as signal regulatory protein a (SIRPα), as the first molecule that was deemed crucial for osteoclastogenic fusion. MFR/SIRPα and its ligand, CD47, both belong to the immunoglobulin superfamily (IgSF). While the expression of CD47 is ubiquitous, MFR/SIRPα is restricted to myeloid cels and neurons. Also, studies have found that the expression of MFR/SIRPα is induced strongly (but transiently) on macrophage lineage cells at the onset of cell fusion, while the expression of CD47 remains constant. It is believed that CD47 initially binds to a form of MRF/SIRPα that has a long extracellular domain, and then switches to binding to a smaller form of MRF/SIRPα following recognition. It is thought that this switch results in bringing the plasma membranes of the homotropic cells close enough for plasma membrane fusion to take place (Vignery, 2005). Studies have suggested that the MRF/SIRPα – CD47 interaction is also involved in cell recognition before the actual act of cell fusion, and that the MFR/SIRPα– CD47 interaction is essential for the survival of fused macrophages, and therefore essential for osteoclastogenic cell fusion (Han *et al.*, 2000).

DC-STAMP

Dendritic cell-specific transmembrane protein (DC-STAMP) has also been shown to be very important for cell fusion during osteoclastogenesis, and is present in dendritic cells and osteoclasts (Kukita *et al.*, 2004) Studies have shown that DC-STAMP-deficient osteoclast precursors are incapable of undergoing cell fusion, even though they are capable of expressing other

osteoclast-specific molecules such as cathepsin K (Yagi *et al.*, 2005). Like MFR/SIRPα, DC-STAMP is important only for cell fusion but not for osteoclastogenic activity in general, and cells lacking DC-STAMP display greatly reduced resorptive activity.

TM4SF CD9 Protein

CD9 is a member of the transmembrane-4 superfamily (TM4SF), or 'tetraspanin', protein family, whose members characterized by four transmembrane domains and known to be involved in cell-cell fusions. CD9 has been implicated in the regulation of cell motility and cell-cell fusion in several cell types, including the fusion of muscle cells (Tachibana *et al.*, 1999) and the formation of myelinated axons (Ishibashi *et al.*, 2004). While details of the involvement of tetraspanins like CD9 are still very vague, studies have shown that the functional blockage of CD9 by neutralizing antibodies results in the inhibition of cell fusion. However, the forced expression of CD9 in monocyte-lineage cells has been shown to result in spontaneous fusion without the expression of osteoclast-specific molecules such as TRAP and cathepsin K. It has therefore been suggested that CD9 may be a downstream molecule of the RANKL signaling pathway (Ishii and Saeki, 2008). One hypothesis has RANKL signaling resulting in the lipid-raft localization of CD9 from the non-raft fraction, followed by CD9 -mediated signal transduction in *cis* with other partner proteins that results in the release of membrane fusion-stimulating signals such as Ca^{2+} elevation (Ishii and Saeki, 2008).

1.3.1.3 Osteoclast Attachment

In a prior chapter, we discussed the prevailing hypothesis regarding the triggering of osteoclast recruitment following the formation of micro-cracks in bone. Upon reaching the site of resorption, the attachment phase of the resorption cycle begins. Osteoclast resorption requires that a strong attachment be made between the cells and the bone surface. However, osteoclasts are highly motile, and the molecular system controlling the dynamic osteoclast-matrix interactions is believed to be an important determinant of the initiation of bone resorption (Baron *et al.*, 1993). Blavier and Delaissé (1995) were the first to suggest that matrix metalloproteinases (MMPs) were critical to the access of osteoclasts to their future sites of resorption after they found that MMP inhibitors completely prevented the formation of the marrow cavity of primitive long bones. In the presence of MMP inhibitors, osteoclasts were not recruited into the core of the diaphysis and instead remained in the surrounding mesenchyme. The effect of the MMP inhibitors on migration, and not resorption, was clarified by the fact that strong inhibitors of resorption such as bisphosphonates and cathepsin K inhibitors did not inhibit the migration of osteoclasts into the diaphysis. More specifically, studies have shown that MMP-9 and MMP-14 deficiency slowed down migration dramatically while MMP-12 deficiency did not affect the recruitment of osteoclasts into the core of the diaphysis of primitive long bones (Shipley *et al.*, 1996; Engsig *et al.*, 2000a; Engsig *et al.*, 2000b).

After migration of an osteoclast to a site of resorption, a sealing zone is formed under the osteoclast in order to seal the resorption site from its surroundings. The sealing zone is formed by the tight attachment of the plasma membrane of the osteoclast to the bone matrix (Väänänen and Horton, 1995).

The osteoclast first organizes its cytoskeleton in such a way that podosomes are gathered to form a circular structure in the plasma membrane facing the bone matrix. These podosomes mediate the attachment of osteoclasts to the extracellular bone matrix via αvβ3 integrin (Marchisio *et al.*, 1988; Lakkakorpi *et al.*, 1991). The podosomes then start to disappear as F-actin begins to form a dense ring-like structure near the osteoclast's bone-facing plasma membrane. Actin-associated proteins like vinculin and talin are then arranged around the actin ring, and subsequently the tight co-localization of F-actin and the αvβ3 integrin seen in the podosomes disappears (Lakkakorpi *et al.*, 1991). The role of αvβ3 integrin in the formation of the sealing zone has been a topic of debate for a number of years. According to Väänänen and Laitala-Leinonen (2008), light and electron microscopic observations have suggested that αvβ3 integrin mediates the attachment of podosomes to the matrix. However, they found that in the final stages of the formation of the sealing zone by the dense actin ring, the αvβ3 integrin moved to the edges of the tight sealing zone. Comparing this finding to that of Lakkakorpi and Väänänen (1991), it is suggested that some other integral membrane protein interaction is formed to connect the cytoskeleton to the extracellular matrix. Chabadel *et al* (2007) have since confirmed this observation. They found that the cores of the podosomes are linked with CD44 and αvβ3 remains associated with a 'cloud' of diffuse actin. These results suggest that αvβ3 integrin is involved in the early arrangements of the cytoskeleton and continues to be a very important signaling pathway between the extracellular matrix and the cytoskeleton. As described by Duong *et al.* (1998), several other proteins such as tyrosine kinase PYK-2 also become localized to podosomes,

suggesting that the formation of the sealing zone appears to also be PYK-2 dependent (Lakkakorpi *et al.*, 1999).

While we can summarize that $\alpha v\beta 3$ integrin activation results in the triggering of an intracellular signaling cascade resulting in the formation of podosomes, it remains to be seen what type of intracellular vesicles mediate the vesicular trafficking needed for the formation and maintenance of the sealing zone in osteoclasts (Väänänen and Laitala-Leinonen, 2008).

1.3.1.3.1 Osteoclastic Bone Resorption

As described by Väänänen (2005), bone resorption involves the dissolution of the crystalline calcium phosphate or hydroxyapatite followed by the degradation of the fibrillar collagen in the extracellular resorption lacunae. These processes require the secretion of a very large about of acid and proteolytic enzymes in to the resorption lacunae, and also demand endocytosis and further transcytosis of the degradation products from the lacunae.

The first step in this complicated cellular process is the formation of the primary organ of resorption, the ruffled border membrane. When an osteoclast is recruited to the site of resorption and is induced to resorb bone, a large number of intracellular acidic vesicles are fused to the bone-facing plasma membrane inside the actin ring (Palokangas *et al.*, 1997). During this fusion of late endosomal vesicles to the plasma membrane, much of the internal membrane is transferred and forms long finger-like projections that penetrate the bone matrix. These acidic vesicles are one source of the acid being released into the resorption space to dissolve the hydroxyapatite crystals ($[Ca_3(PO_4)_2]_3Ca(OH)_2$). The process of the formation of the ruffled border membrane, according to (Zhao, *et al.*, 2001), is dependent on the function of the small GTP-binding protein rab7.

This GTPase is known to regulate trafficking between the early and late endosomes (Spang, 2004) in other cells. In osteoclasts, this GTPase is localized at the ruffled border. Studies now show that rab 7 is restricted only to a certain area of the ruffled border (Mulari, *et al.*, 2003). This area corresponds to the region of localization of vacuolar ATPase and co-localization of cathepsin K in resorbing cells.

At the same time that the acidic vesicles are fused to the plasma membrane, proton pumps are inserted into the ruffled border membrane so that they can continually pump protons via the ATP-consuming proton pump called vacuolar ATPase (V-ATPase) directly from the cytoplasm into the resorption lacuna (Blair, *et al.*, 1989; Tukkanen and Väänänen, 1986; Väänänen *et al.*, 1990). The secretion of protons is accompanied by the counter-movement of chloride anions via a specific chloride channel, namely chloride channel 7- also known as CIC-7 (Kornak, *et al.*, 2001). V-ATPases are electrogenic proton pumps that are present both in a number of various intracellular vesicles as well as in the ruffled border of osteoclasts (Blair *et al.*, 1989; Väänänen *et al.*, 1990; Becker and Gay, 1990). The membrane-bound V-ATPase is composed of at least 5 different subunits, and the soluble catalytic complex contains at least 8 different subunits. In addition, each V-ATPase pump contains several copies of each subunit, and two or more isoforms for each subunit have also been described. These isoforms are very specific to certain cells and intracellular compartments, and therefore there is very high cell and tissue specificity (Drory and Nelson, 2006). The ion equilibrium in the cytoplasm is maintained by the continuous production of protons and bicarbonate ions from carbonic anhydrase II. This is facilitated by CO_2 hydration and the exchange of bicarbonate ions with chloride ions by an HCO_3^-/Cl^- exchanger that is located at the basolateral

membrane. As result, the potential intracellular alkanity induced by the proton transport is prevented (Schlesinger *et al.*, 1997). In order to power the proton pumps and vesicular trafficking, there needs to be a large number of mitochondria so as to ensure high levels of ATP and GTP.

According to Väänänen (2008), it is not presently known which amongst the two main methods of acidifying the resorptive space, namely: (a) the fusion of pre-loaded acid vesicles and the release of their contents directly into the resorptive lacuna, and (b) the insertion of acid secreting molecules in the ruffled border that continuously secrete acid from the cytoplasm into the lacuna, are quantitatively more important for acid secretion into the resorptive space. The author goes on to discuss how these processes are difficult to study because current technology does not allow for good separation of the two phenomena.

The acidity within the resorptive space dissolves the mineral phase, exposing the collagen-rich organic matrix of the bone for the proteolytic enzymes to do their job. Presently, the two main groups of enzymes thought to be responsible for the extracellular degradation of the organic matrix are matrix metalloproteinases (MMPs) and lysosomal cathepsins. There is very good evidence that now suggests that the enzyme cathepsin K is a key ingredient contributing to the degradation of bone matrix in the resorption lacuna. Cathepsin K is expressed at very high levels in osteoclasts and as described by Everts *et al.* (2006) and Troen (2006), is secreted into the resorption lacuna. Cathepsin K has the unique ability to cleave the native triple helix of type I collagen at multiple sites, resulting in the unwinding of the triple helix and thus making it readily available for degradation by any proteinases that have gelatinolyic activity (Delaissé *et al.*, 2003). The deletion of the cathepsin K gene in mice leads to osteopetrosis (Bossard *et al.*, 1996; Saftig *et al.* 1998; Gowen *et al.*, 1999) and

mutations in human cathepsin K lead to pycnodysostosis (Gelb *et al.*, 1996; Johnson *et al.*, 1996). Kiviranta *et al.* (2005) and Zenger *et al.* (2007) recently confirmed that defective bone resorption in cathepsin K–deficient mice is partially compensated for by enhanced osteoclastogenesis. Proteomic analysis of lysosomal acid hydrolases secreted by RAW 264.7 cell derived osteoclasts indicated that these cells secreted almost all known cathepsins, and while the role of cathepsin K in bone resorption is well known, the same cannot be said about the other cathepsins (Czupalla *et al.*, 2006). As described by Väänänen and Laitala-Leinonen (2008), osteoclasts are also very rich in acid phosphatases such as tartrate resistant acid phosphatase (TRAP), which is used as a cellular marker for osteoclasts. The serum levels of the osteoclast–specific TRAcP5b correlates with resorption activity and this can be used as a clinical marker for various resorptive conditions (Halleen *et al.*, 2006). TRAP has two distinct enzymatic activities: it functions as a phosphatase and it can also generate reactive oxygen species (ROS) via the Fenton reaction (Vänänen and Laitala-Leinonen, 2008). Cathepsin K and TRAP are both localized together with bone degradation products in the same transcytotic vesicles, and it is hypothesized that it is here that cathepsin K activates TRAP by limited proteolytic cleavage (Ljusberg *et al.*, 2005; Vaanraniemi *et al.*, 2004). The generation of ROS by TRAP has been indicated in the degradation of collagen, and thus may have a role in the final degradation of resorption products intracellularly (Halleen *et al.*, 2003). This is interesting, as TRAP is also found in dendritic cells, and so Vänänen and Laitala-Leinonen (2008) speculate that there may be a role for TRAP in antigen processing and that its function in osteoclasts is more to reduce any risk of autoimmunity against bone proteins than to have a functional role in bone resorption.

The role of matrix metalloproteinases (MMPs) in bone resorption, in contrast to that of cathepsin K, is still being debated. As described by Delaissé *et al.* (2003), MMPs are extracellular proteinases that are produced in the latent form and their activation often occurs through activation cascades involving other MMPs. MMPs have a catalytic domain as well as several other domains that determine the specificity of each MMP towards substrates and regulators. There are several MMPs that are expressed in bone but only some of them – including MMP 9 and MMP 14, are produced by osteoclasts (Andersen *et al.*, 2004; Linsuwanont-Santiwong *et al.*, 2006). Fuller *et al.* (2007) conducted a study that showed only a minimal effect of MMP inhibitors on collagen degradation. Some other MMPs that have been shown to solubilize collagen include interstitial collagenases such as MMP-13 and MMP-14, gelatinases such as MMP-2 and MMP-9, and other MMPs such as MMP-12. Several studies conducted by Tezuka *et al.* (1994) and Sato *et al.* (1997) show that none of the MMPs of osteoclastic origin were limiting for resorption, whereas MMP-13 proved to be limiting for resorption. MMP-13 does not originate from osteoclasts, but from osteocytes (Fuller and Chambers, 1995; Shimizu *et al.*, 1990) or mononuclear cells that surround osteoclasts (Fuller and Chambers, 1995).

Removal of degraded hydroxyapatite from Resorption Lacunae

The acid-driven dissolution of apatite crystals results in the release of a very large quantity of calcium and phosphate from the solid mineral phase that needs to be transported away from the site of resorption in order to ensure the continuation of the resorption process. In addition to removing the products of acid dissolution, there is also the need to remove collagen fragments that have been generated by cathepsin K in the lacuna. Studies (Nakamura *et al.*, 1995;

Takeshita *et al.*, 2002) have shown that degradation products are targeted to a specific plasma membrane domain in the upper part of resorbing osteoclasts known as the functional secretory domain (FSD). The degradation products are endocytosed through the cell, and then finally secreted into the extracellular fluid through the FSD. The transcytotic pathway finding is supported by studies that found thick bundles of microtubules that originate from the ruffled border and continue towards the FSD lend further support to the transcytotic pathway (Hotozekaka *et al.*, 2002; Mao *et al.*, 2006). Also, Nakamura *et al.* (1995) conducted an elegant study wherein they biotinylated the surface of bone slices and then studied their subsequent liberation by osteoclastic resorption. They followed the movement of these biotinylated proteins through the osteoclast using fluorescence microscopy and found that the proteins were concentrated at the basolateral membrane. In addition, their studies revealed that there was a local loss of cortical F-actin in the areas of biotin accumulation – an observation that is in line with results that show local disassembly of the cortical F-actin network during exocytosis in mast cells, chromaffin cells, and pancreatic acinar cells (Koffer *et al.*, 1990; Vitale *et al.*, 1991; Muallem *et al.*, 1995).

There is also a release of bicarbonate ions from the breakdown of hydroxyapatite. Bicarbonate would rapidly neutralize at low pH and thereby prevent further demineralization if it is not removed. Laitala-Leinonen's group conducted studies that provided some insight into how excess bicarbonate is removed from the resorption lacuna. They showed that the electroneutral sodium-bicarbonate co-transporter NBCn1 is found extensively in the ruffled border of resorbing osteoclasts, and that its proper function is to ensure normal osteoclast function. Even if the buffering capacity of solubilized hydroxyapatite could be overcome by the removal of excess bicarbonate, the resorption process

would still not progress until the calcium and phosphate ions are removed from the lacuna. According to Väänänen and Laitala-Leinonen (2008), this major flow of ions is still very poorly understood and a very little studied phenomenon. They suggest that in theory, there are at least three different mechanisms for calcium and phosphate removal from the lacuna. These include transcytotic transport of molecules in the membrane vesicles from the ruffled border to the functional secretory domain of the osteoclast, the transient release of ions beneath the sealing zone, and finally the transport of ions through the cell using calcium/phosphate binding proteins and ion channels or pumps at the plasma membrane.

Studies conducted by Yamaki *et al.* (2005) suggest that calcium is transported in the same transcytotic vesicles that carry collagen and other matrix protein fragments from the ruffled border to the functional secretory domain. There has also been evidence suggesting that osteoclasts can efficiently transport calcium from the lacuna via an intracellular pathway that utilizes TRPv5, one of the most Ca^{2+} selective transient receptor potential (TRP) channels. Van der Eerden *et al.* (2005) found that deleting TRPv5 resulted in the abolition of osteoclast activity. Väänänen and Leitala-Leinonen (2008) suggest that it is probably that both vesicle-mediate transcytosis and active transcellular transport are needed in order to handle the large quantities of calcium in a short period of time. There is currently no evidence of any mechanism by which calcium and other soluble substances are released beneath the sealing zone directly into the extracellular medium.

In the case of phosphate removal, there is no evidence of vesicle-mediated transcytosis but recent studies suggest the presence of transcellular transport of phosphate (Gupta *et al.*, 2001). However it is not yet known how

large the quantities of phosphate being transferred are, so all three routes of phosphate disposal should still be considered-namely, vesicle-mediated transcytosis, specific transcellular transport, and leakage beneath the sealing zone.

1.3.1.4 Transition Phase

The transition phase of bone remodeling is a critical period for the 'coupling' of bone resorption and bone formation. This is the phase during which bone-resorbing osteoclast stimulate the differentiation of osteoblast precursors, thereby promoting bone formation in the pits formed by bone resorption. While bone formation is being activated, osteoclastic bone resorption is halted and osteoclasts undergo apoptosis either in a Bim/caspase-3-dependent manner (Wakeyama *et al.*, 2007) or through estrogen-induced Fas ligand (Nakamura *et al.*, 2007). It is believed that the high extracellular calcium content that results from bone resorption is responsible for the induction of osteoclastic apoptosis (Zaidi *et al.*, 1989; Lorget, *et al.*, 2000; Nielsen, *et al.*, 2007). The transition from bone resorption to bone formation happens as a result of coupling factors that are either liberated, secreted, or membrane-bound.

- **Liberated coupling factors**

The activation of osteoblastic bone formation is a critical step in coupling, and can occur with or without direct cell-to-cell contact. Osteoclastic bone resorption may result in the liberation of growth factors such as TGF-β, bone morphogenic proteins (BMPs), and insulin-like growth factor (IGF)-II from the bone matrix (Pfeilschifter and Mundy, 1987; Wozney *et al.*, 1988) These would then activate osteoblastic bone formation. Studies have also shown that it

is the number of osteoclasts, and not necessarily the act of resorption, which is critical for the stimulation of bone formation (Del Fattore *et al.*, 2006; Karsdal *et al.*, 2005).

- **Secreted coupling factors**

As described by Gray *et al.* (1996), the local topography of a site of resorption, such as the grooves and pits, play a part in inducing bone formation by enhancing cellular condensation. Alternatively, is its also possible that osteoblasts may in fact recognize components of previously resorbed lacunae in the absence of osteoclasts. For example, Type V tartrate-resistant acid phosphatase (TRAP, encoded by *Acp5*) is required for bone matrix resorption and collagen turnover, but transgenic mice over-expressing TRAP show not only increased loss of trabecular bone volume but also show increased bone formation (Angel *et al.*, 2000). This suggests that TRAP, which usually helps with resorption in the lacunae, may in fact have a hand in stimulating bone formation. Several *in vitro* studies suggest that osteoclasts secrete potential coupling factors, including sphingosine 1-phosphate (S1P), a B polypeptide chain platelet-derived growth factor homodimer (PDGF BB), and hepatocyte growth factor (HGF). S1P is produced in osteoclasts by sphingosne kinase (*Sphk*) and can act as both an intracellular and extracellular messenger. Secreted S1P interacts with its receptor on osteoblasts and enhances RANKL expression as well as migration and survival of osteoblasts (Ryu *et al.*, 2006). PDGF BB promotes proliferation but suppresses differentiation of osteoblast precursors (Kubota *et al.*, 2002; O'Sullivan *et al.*, 2007). So when osteoclasts undergo apoptosis, the reduced PDGF BB secretion is thought to contribute to the switch from proliferation to differentiation in osteoblasts. HGF, which is secreted by

osteoclasts and not osteoblasts, binds to HGF receptors expressed on both cell types (Grano *et al.*, 1996). HGF treatment increases DNA synthesis and proliferation of both cell types.

- **Membrane-bound coupling factors**

Osteoclast inhibitory lectin (OCIL) is a type II transmembrane C-type lectin that suppresses osteoclast differentiation (Zhou *et al.*, 2001; Zhou *et al.*, 2002). OCIL has been shown to inhibit osteoclast differentiation and function *in vitro* (Nakamura *et al.*, 2007). Steel factor (SLF) - which is also known as stem cell factor (SCF) and mast cell growth factor (MGF), is the ligand for the tyrosine kinase receptor c-Kit (also known as CD117). A mutation that results in the formation of soluble SLF has been shown to result in osteopenia, impaired osteoblast function, and increased osteoclast surface, suggesting that it is the membrane-bound and not the soluble SLF that suppresses osteoclast formation and enhances bone formation by osteoblasts (Lotinun *et al.*, 2005).

- **Direct OC-OB contact *in vivo***

In 2002, Everts *et al.* reported that the proteolytic removal of bone collagen left by osteoclasts in the resorption lacunae is an essential step linking bone resorption to bone formation. The authors found that almost all of the osteoclasts attached to the bone surface were in contact with bone lining cells. Matuso and Irie (2008) suggest that it is possible that mature resorbing osteoclasts interact not only with bone lining cells, but also with fully differentiated osteoblasts. They cited their own preliminary observations that used transmission electron microscopy to detect cell-cell contact between mature osteoclasts and mature osteoblasts.

1.3.1.5 Hormones that Control Bone Resorption

1.3.1.5.1 VITAMIN D

Vitamin D is synthesized following exposure to sunlight and can also be acquired from the diet (DeLuca, 2004). Vitamin D, which according to Haddad (1995) is bound to the vitamin D-binding protein (DBP), is then transported to the liver to be converted to the active form of 1,25-dihydroxyvitamin D (1,25-$(OH)_2$ D) by cytochrome P450 25-hydroxylase enzymes (Cheng *et al.*, 2003; Ohyama and Yamasaki, 2004). Studies by (DeLuca, 1988) discovered that one of the major physiological roles of 1,25-$(OH)_2$ D, also known as calcitriol, is in the maintenance of mineral homeostasis. Researchers have found that in animals, vitamin D deficiency results in conditions such as hypocalcemia, secondary hyperparathyroidism, rickets, and osteomalacia. 1,25-$(OH)_2$ D has been shown to play a crucial role in the intestinal absorption of calcium by inducing the expression of epithelial calcium channels (Van Cromphaut *et al.*, 2001). In addition to this, 1,25-$(OH)_2$ D induces the transcription of genes that code for proteins such as calbindin D9k and the basolateral membrane pump PMCA1b. These proteins facilitate the movement of calcium through the enterocyte cytoplasm and transport the calcium across the basolateral membrane and into circulation (Christakos *et al.*, 2003; Raval-Pandya *et al.*, 1998; Wasserman *et al.*, 1992).

VDR is expressed on osteoblasts and precursors, and studies have shown that the 1,25 $(OH)_2$ D can modulate the expression of osteoblastic phenotype markers (Owen *et al.*, 1991) depending on the growth and maturation stage of the cells. As explained by St. Arnaud (2008), VDR signaling may inhibit differentiation in the early part of osteoblastogenesis, but VDR signaling in terminally differentiated osteoblasts results in enhanced osteoblastic activity.

1.3.1.5.2 PARATHYROID HORMONE

The parathyroid hormone (PTH) is synthesized in the parathyroid glands, and in mammals is the most important regulator of blood calcium ion concentrations. The synthesis and secretion of PTH is very finely regulated by the concentrations of extracellular calcium ions, as monitored by calcium-sensing receptors present on surface of parathyroid gland cells. As described by Gardella and Jüppner (2000), the release of PTH requires only very minor reductions in the circulating levels of calcium, and it acts by activating PTH-1 receptors expressed in bone and kidney tissues. The PTH-1 receptor belongs to the G-protein coupled receptor family, and the triggering of the receptor by PTH results in the downstream production of cAMP and the activation of protein kinase A (PKA). This results in the release of calcium from bone and stimulates the production of 1,25-(OH)$_2$ D, as well as increasing the retention of calcium from the glomerular filtrate (Gardell and Jüppner, 2000).

1.3.1.5.3 ESTROGEN

As described by Raisz (2005), the initial idea that estrogen deficiency is critical to the pathogenesis of osteoporosis was based on the fact that post-menopausal women, whose estrogen levels decline naturally, are at the highest risk of developing osteoporosis. Studies have found that the biochemical markers of both resorption and bone formation are increased following menopause, suggesting that bone remodeling is accelerated at that stage in life (Parfitt *et al.*, 1995; Ebeling *et al.*, 1996). Estrogen acts through two estrogen receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ), with ERα appearing to be the primary mediator of estrogen's actions on the skeleton (Lee *et al.*, 2003). The human estrogen receptor belongs to the nuclear receptor super-family of

ligand-inducible transcription factors (Evans, 1988). In the absence of the hormone, the estrogen receptor is sequestered within the nuclei of target cells in a multi-protein inhibitory complex. Once the ligand binds the receptor, there is a conformational change within the estrogen receptor that promotes homo-dimerization and high affinity binding to specific DNA response elements located within the regulatory regions of the target genes (Tsai and O'Malley, 1994). ERα, ERβ, and the androgen receptor (AR) are all present in bone marrow stromal cells, osteoblasts, and osteoclasts and their progenitors (Bellido *et al.*, 1995; Couse and Korach, 1999). This suggests that the effects of sex steroids on bone are mediated, to some extent, directly. Receptor expression levels in bone cells is about 10-fold lower than that found in reproductive organs, and does not vary by gender as similar levels of ER and AR have been found in both men and women (Benz *et al.*, 1991; Braidman *et al.*, 2000).

Effects of Estrogen on Bone Cells

Estrogen tends to have an anabolic effect on isolated osteoblasts, and is complemented by its inhibition of apoptosis in these cells (Van Pottelbergh *et al.*, 2004). Estrogen also suppresses osteoclast activity via increased osteoclast apoptosis (Albagha *et al.*, 2005), reduced osteoblastic production of RANKL, and by increased production of OPG (Khosla *et al.*, 2004). These direct effects are further bolstered by estrogen's ability to reduce the production of cytokines.

Sex hormones and Cytokines

TGF-β

In 1996, Hughes *et al.* proposed a major role of estrogen before menopause is the limitation of osteoclast numbers and activity through the promotion of apoptosis of mature osteoclasts and their precursors. They suggested that TGF-β is responsible for triggering apoptosis in osteoclasts and that it also mediates the effects of estrogen and tamoxifen. They found that TGF-β increased in osteoclast apoptosis in a dose-dependent manner, and that apoptosis was blocked with the introduction of an anti-TGF-β antibody. They also found that this antibody inhibited estrogen and tamoxifen-induced osteoclast apoptosis, a finding that furthered strengthened their theory regarding the relationship between estrogen, TGF-β, and osteoclast apoptosis.

IL-1, IL-6, and IL-7

Studies have shown that there is an increase in the levels of interleukin-1 (IL-1) and interleukin-6 (IL-6) following menopause, and are the first cytokines to appear and be responsible for bone loss from estrogen deficiency. As described by Manolagas (1995) and Manolagas *et al.* (2002), high levels of IL-6 have been associated with increased osteoclastogenesis, further confirming the role of cytokines in bone loss. The authors found that estrogen and androgen suppress the production of IL-6 as well as two subunits of the IL-6 receptor in cells of bone marrow stromal/osteoblastic lineage. The case for a role for IL-6 in bone loss is further confirmed by a study conducted by Bellido *et al.* (1995), where they found that IL-6 knock- out mice did not experience bone loss following the loss of sex steroids. Recently, IL-7 has also been linked to post-menopausal bone loss. It

has been found that IL-7 levels are significantly upregulated following ovariectomy, and the *in vivo* neutralization of IL-7 completely prevents ovariectomy-induced bone loss in mice. In addition, suppressing the rise in levels of IL-7 following ovariectomy also leads to upregulated bone formation (Weitzmann *et al.*, 2002).

1.3.1.5.4 CALCITONIN

The hypocalcemic peptide calcitonin (CT) was discovered by Copp *et al.* in 1962 and contributes to calcium homeostasis by direct inhibition of osteoclasts (i.e., bone resorbing cells). CT also reverses the process of osteolytic osteolysis by promoting a flux of calcium into the bone. In addition, CT enhances calcium excretion from the kidneys (Friedman and Raisz, 1965; Warshawsky *et al.*, 1980). As described by Pondel in 2000, studies that were carried out to determine the source of CT have resulted in the realization that there are multiple types of CT present within one species, and its expression ranges from the brain to the prostrate.

Synthesis and Structure of Calcitonin (CT)

The calcitonin (CT) gene complex consists of two known α and β genes, both of which are located between the catalase and parathyroid hormone genes on chromosome 11 in humans (Kittur *et al.*, 1985). The α calcitonin gene consists of six exons, of which the first three are shared with the alternative splice product, calcitonin gene-related peptide (CGRP). The β calcitonin gene is similarly organized, but has significantly different 3' and 5' noncoding regions (Alevizaki *et al.*, 1986) and gives rise to β-CGRP. The α gene upon splicing at exon 4 gives rise to calcitonin, and produces the neuropeptide and potent

vasodilator CGRP when spliced at exon 6 (Emson and Zaidi, 1989). The first three exons are spliced in either process, and so both products show the same 5' coding and noncoding regions. Lou *et al.* (1994) and Zandberg *et al.* (1995) have identified important regulatory elements in exon 4 that are likely involved in directing splicing towards CT or CGRP. There is also an intron element that resides in intron 4 of the CT gene (Lou *et al.*, 1996) that is possibly involved in tissue-specific regulation of calcitonin RNA processing (Lou and Gagel, 1998). As described by Monla *et al.* (1995) and Peleg *et al.* (1993), the calcitonin promoter possesses a negative response element to vitamin D, a cyclic-response element (CRE) and a transcriptionally active octomer sequence (CRE-O). Following splicing to either CT or CGRP mRNA, mature peptides are synthesized initially as large precursors that are then cleaved intracellularly to release the active molecule.

The mature CT peptide is 32 amino acids long, and contains an N-terminal disulfide bridge between residues 1 and 7 (Zaidi *et al.*, 1990) as well as a proline amide at residue 32. The CT sequence varies between species, and it has been suggested that the conformational flexibility of a given CT molecule – which is dependent on the bulkiness of the relevant side chains, determines its biological potency (Breimer *et al.*, 1998). As a result, the less bulky eel and salmon CTs renders them more potent than human CT (Zaidi *et al.*, 2002).

Calcitonin Receptor (CTR)

The physiological effects of CT (inhibition of osteoclast-mediated bone resorption; increased excretion of Ca^{2+} from the kidneys) are mediated by high affinity calcitonin receptors (CTRs). According to Abou-Samra, the calcitonin receptor is closely related to the receptors for secretin and the parathyroid

hormone (PTH), and along with them forms a distinct subfamily within the G-protein coupled receptor superfamily. G-protein coupled receptors (GPCRs) represent the largest family of signal-transducing molecules known (Gersenghorn *et* al., 2001). A large number of hormones, neurotransmitters and other ligands exert their effects on cells by binding to G-protein coupled receptors. The chemisty of ligands for the different GPCRs vary remarklably, and range from biogenic amines and glycoproteins to lipids and ions to name a few.

GPCRs were named based on their ability to recruit and regulate the activity of intra-cellular G-proteins. Following binding, heterotrimeric G-proteins transduce the ligand binding into intracellular responses that result in physiological responses of tissues and organisms. Initially, G proteins are bound to GDP and are in an inactive state. When activated GPCRs interact with G proteins, they act as guanine-nucleotide exchange factors and induce a conformational change in the associated G-protein a subunit causing a release of GDP, followed by the binding of GTP (Bourne *et al.*, 1991). The binding of GTP results in the dissociation of the G_{α}- GTP from the $G_{\beta\gamma}$ subunits, and these products can modulate several signaling pathways such as the inhibition/stimulation of adenylate cyclases, phospholipases, as well as calcium channel activity.

G-protein coupled receptors are grouped into three subfamilies that display little sequence homology, and yet share the same overall topology. The only structural feature common to all GPCRs is the presence of 7 transmembrane α helices. There is however a lot of sequence homology between members of the same sub-families. As described by Foord (2002), there are three main sub-families of GPCRs: the rhodopsin receptor-like family (Family

A or Class I), the secretin receptor-like family (Family B or Class II), and the neurotransmitter receptor family (Family C or Class III).

Calcitonin Receptor: A Family B G-protein coupled receptor

The first calctionin receptor (CTR) to be isolated and cloned was derived from a cDNA library from the porcine renal epithelial cell line by Lin *et al.* in 1991, and it was one of the first Family B (or Class II) G-protein coupled receptors to be identified. The most prominent characteristic of this family of receptors is the presence of a very large extracellular amino-terminus containing several cysteines, presumably forming a network of disulfide bridges (Ulrich *et al.*, 1998). Autoradiographic and radioligand-binding techniques using iodinated CT have since identified high-affinity CTRs in a variety of tissues including primary breast cancer tissue (Gillespie *et al.*, 1997), kidneys, discrete regions of the brain and placenta (Martin and Moseley, 1990).

Calcitonin and Cell Signalling

The physiological effects of CT are mediated mainly by the ability of the CTR to couple to at least two signal transduction pathways. The coupling of the CTR to the activation of adenylate cyclase/cAMP/protein kinase A pathway has been described in several cell types – for example, kidney epithelial cells following treatment with soluble CT (Goldring *et al.* 1978). In 2000, Orcel *et al.* constructed CTR hybrid insulin-like growth factor II receptors and transfected them into COS cells to identify G-protein-interacting domains of the pCTR. Two regions of the pCTR were demonstrated to be crucial for interaction with G_s, and subsequently with increased intracellular levels of cAMP. As stated by Chabre *et al.* and Force *et al.* in 1992, CTRs can also couple to the phospholipase C

enzyme pathway. This pathway, as with the cAMP pathway, can be initiated by the coupling of receptors to multiple G-proteins. Activation of the PLC pathway causes the release of Ca^{2+} from intracellular stores and helps promote an influx of extracellular calcium. Finally, in 1998, Naro *et al.* suggested that CTRs can also couple to the phospholipase D signal transduction pathway. While it is established that adenylate cyclase, PLC, and PLD are all signal effectors for the CTR, there appear to be more signalling pathways that have been less extensively characterized. For example, CTR-mediated activation of mitogen-activated protein kinase (MAPK) pathway was described by Gutkind *et al.* in 1998.

Actions of CT

According to Breimer *et al.* (1988), calcitonin inhibits both basal and stimulated resorption of organ-cultured bone. It functions by causing a loss of the ruffled border of osteoclasts (Chambers and Magnus, 1982; Holtrop *et al.*, 1974; Kallio *et al.*, 1972) and results in a decrease in osteoclasts numbers over the course of time. Studies by Chambers (1982) have shown that the application of near-physiological femtomolar calcitonin concentrates is enough to stop cytoplasmic motility of osteoclasts *in vitro*, and even produces a gradual retraction of isolated osteoclasts. In addition to this, calcitonin inhibits osteoclasts from synthesizing and secreting degradative enzymes such as TRAP (Yumita *et al.*, 1991). Calcitonin has also been shown to reduce the amount of acid secreted by osteoclasts by altering Na^+-K^+-ATPase activity and carbonic anhydrase localization (Akisaka and Gay, 1986; Chambers *et al.*, 1987; Moonga *et al.*, 1990). The different forms of calcitonin display different efficacies in terms of the inhibition of osteoclast activity, with salmon calcitonin being the most potent and

human calcitonin being the least potent (Zaidi *et al.*, 1988). One of calcitonin's most pharmacologically important functions is to increase renal calcium and phosphate excretion as well as increasing 1,25-$(OH)_2$ D production (Agus *et al.*, 1981; Zaidi *et al.*, 1990). Calcitonin has also been shown to regulate the expression of the renal 25-hydroxyvitamin D3 hydroxylase gene in rats (Shinki *et al.*, 1999). As described by Nicholson *et al.* (1986), there are about 1 million calcitonin receptors on osteoclasts, and several receptor subtypes that have been formed as a result of alternative splicing have been identified (Goldring *et al.*, 1993). It is important to note that all of the subtypes bind calcitonin with high affinity. Other interesting studies have found that M-CSF and NFκB regulate the expression and function of the calcitonin receptor in human osteoclast-like cells (Samura *et al.*, 2000) and treatment of osteoclasts with CT induced a prolonged but temporary down-regulation of CTR expression (Wada *et al.*, 1995). Inoue *et al.* (1999) and Wada *et al.* (1997) studied the molecular basis of CTR gene expression and suggested that the CT inhibited transcription of CTR mRNA by inhibiting its transcription or by reducing its stability, respectively.

1.4 OSTEOPOROSIS THERAPY STRATEGIES

As discussed earlier, osteoporosis is a bone-degenerative disease that is brought about by an imbalance in bone remodeling that favours bone resorption over bone formation, eventually resulting in severe bone fragility. Therefore, treatments for osteoporosis can be either anabolic therapies (eg. PTH) or anti-resorptive therapies (eg. Bisphosphonates, CT).

1.4.1 ANABOLIC THERAPIES

Anabolic therapies are those therapies that increase bone mass and strength, and primarily work by stimulating bone formation. As described by Lane and Kelman (2003), this new class of agents increases bone mass to a greater extent than what is seen by simply filling in resorption pits. As a result, the authors suggest that these drugs have the potential to restore bone mass back to normal levels, thus reducing the risk of osteoporotic fractures by more than the currently available antiresorptive agents.

1.4.1.1 Parathyroid Hormone (PTH)

The anabolic effects of PTH have been discussed in detail in a previous chapter, and Silverman *et al.* in 2001 showed that the aminoterminal fragment of PTH (also known as teriparatide) was able to produce significant reductions (65% and 53%) in the risk of vertebral and non-vertebral fractures. Treatment with the full length PTH was shown to reduce risk of new vertebral fractures by 58%, and the increase in BMD obtained is further amplified when a bisphosphonate is given after a course of PTH. Currently, recombinant human PTH is approved as a therapy for postmenopausal osteoporosis in the US (Lane and Kelman, 2003).

1.4.1.2 Strontium Ranelate

Strontium ranelate consists of two cation atoms of stable strontium and an organic moiety, ranelic acid, which dissociates at the gastrointestinal level. Strontium is a cation that is very closely related to calcium and has been shown to play both an anabolic and an antiresorptive role in bone metabolism, although there is still some controversy surrounding its mechanism of action (O'Donnell *et*

al., 2006). Potential side effects, according to O'Donnell *et al.* (2006), include diarrhea, seizures and memory loss.

1.4.2 ANTIRESORPTIVE THERAPIES

Age-related bone loss can be essentially halted by the use of antiresorptive agents to restore the balance between bone resorption and formation (Reid, 2008). The currently available resorptives will be discussed in this section.

1.4.2.1 Selective Estrogen Receptor Modulators (SERMs)

As described in an earlier section, estrogen is an anti-resorptive agent that is crucial to the maintenance of skeletal integrity in adults of both sexes, and acts on cells directly as well as indirectly. The use of estrogen in post-menopausal women has been shown to return bone turnover markers back to premenopausal levels, and if administered at the right time can completely prevent postmenopausal bone loss (Lindsay *et al.*, 1980). When researchers administered estrogen to women who had been postmenopausal for several years, it resulted in an 8% increase of spinal bone mass over the course of 3 years when compared to a placebo (Bush *et al.*, 1996). There was also a one-third reduction in the number of spine and hip fractures (Rossouw *et al.*, 2002). However, there are several safety concerns that have arisen over the years that have substantially limited the use of this therapy.

There has always been a concern regarding the link between estrogen administration and an increased incidence of breast cancer, and this was confirmed by a study by the Women's Health Initiative (Rossouw *et al.*, 2002). Studies by the Women's Health Initiative have found that in treatment with estrogen alone in the presence of a uterus resulted in an increased rate of

endometrial carcinoma, and so this treatment is only an option for women who have had hysterectomies. They then found that treatments that combined estrogen with progesterone resulted in an increased risk of breast cancer. They also found that estrogen treatments increase the risk of thromboembolic events (Rossouw *et al.*, 2002). As a result of these findings, researchers developed estrogen-like agents in an attempt harness that power of estrogen while minimizing its side effects. These compound are known as SERMs, and have a mixture of estrogen agonist and antagonist properties that are tissue-specific (Katzenellenbogen *et al.*, 2002). The first such SERM was tamoxifen, which was initially designed as a receptor antagonist for the treatment of breast cancer. However when it became apparent that it also improved bone density (Grey *et al.*, 1995), it was suggested that it acted as an agonist on bone. This led to the development of raloxifene, a SERM that has less potent effects on bone resorption and bone density than a usual dose of estrogen (Reid *et al.*, 2004). While Ettinger *et al.* (1999) showed that raloxifene resulted in the decreased incidence of vertebral fractures, there was no effect on non-vertebral fractures. In addition, studies by Barett-Connor *et al.* (2006) have found that while raloxifene is neutral with respect to heart attacks and strokes, it does increase the frequency of thromboembolism in the same way that estrogen does. As a result, its use in the management of postmenopausal osteoporosis is limited (Reid, 2008). As described by Maricic (2007), while estrogen is currently approved for the treatment of postmenopausal symptoms, it is no longer approved for the treatment of osteoporosis. Researchers are trying to design SERMs that have more potent bone effects, but most clinical trials have been scratched due to the high incidence of uterine collapse that is seen with these treatments (Reid, 2008). Current SERMs being tested in clinical trials are basedoxifene and

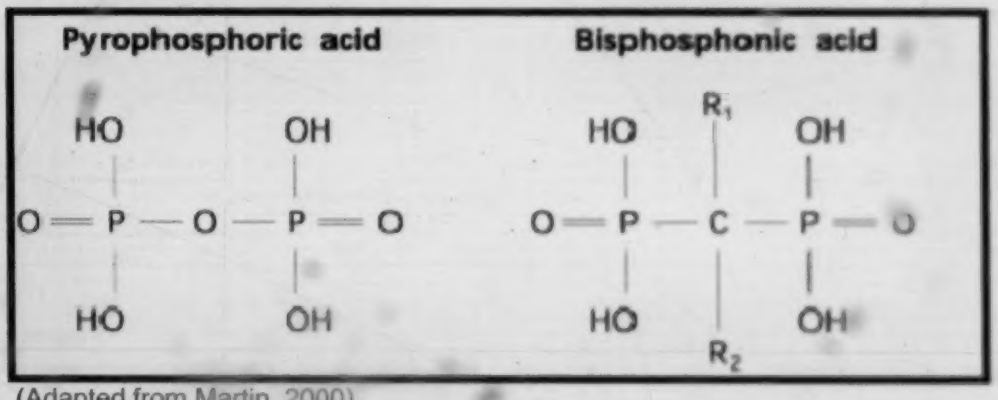
lasofoxifene, but neither is ready as of yet. As a result, SERMs are yet to play a major role in the management of postmenopausal osteoporosis.

1.4.2.2 Calcium

Calcium supplementation is one of the oldest forms of anti-resorptive therapy, and has long been regarded as a 'tonic' for bone. Calcium supplements work by suppressing the circulating levels of PTH, resulting in a decrease in bone turnover (Reid *et al.*, 2008). Following the administration of calcium, the suppression of bone resorption is apparent within a few hours (Reid *et al.*, 1986) and this suppression is sustained with long-term use for at least 5 years (Reid *et al.*, 2006). However, as stated in a review by Reid (2008), one of the issues with having to maintain long-term calcium supplementation is that the compliance levels are low. The reasons for this could be because the tablets are very large and frequently cause gastrointestinal side effects. While calcium supplementation has traditionally been thought of as a safe intervention with the only side effect being constipation, Reid (2008) reported that there was a 50% increase in the occurrence of cardiovascular events following calcium treatments (Bolland *et al.*, 2008). There have also been some other studies that found that when patients with renal failure were randomly administered calcium supplements, there was an increase in vascular calcification and in some cases even resulted in death (Russo *et al.*, 2007; Block *et al.*, 2007). In healthy adults, there have been some studies that found that there was a relation between serum calcium levels and carotid artery plaque thickness (Rubin *et al.*, 2007).

1.4.2.3 Bisphosphonates

Over the last decade, the main class of anti-resorptive agents used in the treatment of osteoporosis has been bisphosphonates. Fleisch *et al.* in 1969 were the first researchers to describe the anti-resorptive effects of this class of drugs, which are analogs of pyrophosphates- the endogenous regulators of mineralization in the body. The only difference is that the phosphate groups in pyrophosphates are joined together by a phosphoanhydride bond (P-O-P) while in bisphosphonates, they are joined together through a central carbon atom resulting in the formation of a phosphoester bond (P-C-P). This P-C-P bond makes bisphosphonates more resistant to enzymatic hydrolysis (Fleisch, 1997), thus allowing them to retain activity *in vivo* over a period of several years. The similarity in the structures of pyrophosphates and bisphosphonates is clearly visible in the figure below:



(Adapted from Martin, 2000)

Fig. 1.1: The structures of pyrophosphate (pyrophosphoric acid) and its analog bisphosphonate (bisphosphonic acid).

As described by Font *et al.* (2008), while the central ring is essential for binding hydroxyapatite, the lateral chains (R1 and R2) are variable and can either

contain a terminal amino chain or a cyclic nitrogen chain. Of the two side-chains, it is understood that the R1 side-chain is usually a hydroxyl group that enhances the affinity of the compound to bone (Widler *et al.*, 2002; Rogers *et al.*, 1999). The potency of the compound as an anti-resorptive is determined by the variations in the structure and conformation of the R2 side-chain (Widler *et al.*, 2002; Rogers *et al.*, 1999; Russell *et al.*, 1999). Based on these side-chains, bisphosphonates can be divided into two main groups: nitrogen-containing/amino bisphosphonates (Alendronate, Risedronate, Pamidronate, Zoledronate, Ibandronate) and non-nitrogen containing bisphosphonates (Etidronate, Tiludronate). A summary of their structures is shown below:

Agent	R_1 side chain	R_2 side chain
Etidronate	-OH	$-CH_3$
Clodronate	-Cl	-Cl
Tiludronate	-H	
Pamidronate	-OH	$-CH_2-CH_2-NH_2$
Neridronate	-OH	$-(CH_2)_5-NH_2$
Olpadronate	-OH	$-(CH_2)_2N(CH_3)_2$
Alendronate	-OH	$-(CH_2)_3-NH_2$
Ibandronate	-OH	
Risedronate	-OH	
Zoledronate	-OH	

Fig.1.2: The structures of the R1 and R2 side-chains of some common bisphosphonates, including the first generation non-nitrogen containing (non-amino) bisphosphonates and the second and third generation nitrogen-containing (amino) bisphosphonates.

Early bisphosphonates were non-aminobisphosphonates, and contained a short R2 side-chain such as a methyl group in etidronate or a chloride group in clodronate. However, they are not as effective as the newer bisphosphosphonates that contain an amino-group in the side chain (Russell *et al.*, 1999). Bisphosphonates like ibandronate even contain a tertiary amino group as their R2 side-chain, and are one of the most potent bisphosphonates as a result (Russell *et al.*, 1999; Gatti, 1999).

All bisphosphonates carry a negative charge that is associated with the phosphate groups, and as a result adhere themselves to the positively charge surface of hydroxyapatite crystals of bone. It has been suggested that approximately 50% of an intravenous dose is sequestered in bone within a few hours of administration and the kidneys excrete the leftover bisphosphonate over a similar time-period. When osteoclasts arrive at their site of resorption and breakdown the mineral phase of bone, they ingest any attached bisphosphonates along with the mineral phase. Upon entering the osteoclast, the bisphosphonates interfere with metabolic pathways that involve any diphosphate moieties. The non-aminobisphosphonates are metabolically converted into non-hydrolyzable analogs of adenosine triphosphate (ATP) by reversing the reactions of aminoacyl-transfer RNA synthetases (Rogers *et al.*, 1994). This leads to an accumulation of resultant metabolites within the osteoclasts, likely resulting in their eventual death (Graham and Russell, 2007). The main pathway inhibited by the more potent class of bisphosphonates, the aminobisphosphonates, is the important biosynthesis pathway, the mevalonate pathway. The final outcome of aminobisphosphonate action is the triggering of apoptosis, an action that Hughes *et al.* (1995) and Selander *et al.* (1996) suggested was the primary means by which amino-BPs inhibit resorption.

The most effective bisphosphonate to date is Zoledronate (or Zoledronic acid), which is a third generation bisphosphonate and is the monohydrate of 1-hydrox-2 bisphosphonic acid (Green and Rogers, 2002).

Bisphosphonate Side-Effects

Several studies have found that oral administration of bisphosphonates causes gastrointestinal inflammation. This is probably as a result of the inhibition of the mevalonate pathway in gastrointestinal epithelial cells (Reszka *et al.*, 2001). The development of gastric ulcers and erosions have is another well-documented side effect associated with the oral administration of nitrogen-containing bisphosphonates (de Groen *et al.*, 1996). Following an intravenous dose of nitrogen-containing bisphosphonates, approximately 20% of patients experienced a temporary flu-like illness also known as acute–phase reaction (APR). It has also been proposed that bisphosphonates cause an excessive reduction in bone turnover that leads to an accumulation of microcracks that make the bone more susceptible to necrosis following trauma or infection (Mashiba *et al.*, 2001). Osteonecrosis of the jaw is a condition that is characterized by necrotic exposed bone in the maxillofacial region, and its connection to bisphosphonates was first reported in patients with cancer that had been receiving intravenous bisphosphonate therapy (Marx, 2003). This condition is not as widely reported by patients with postmenopausal osteoporosis or Paget disease that have been treated with bisphosphonates.

1.4.2.4 Osteoprotegerin (OPG)

As described in an earlier chapter, OPG is a potent inhibitor of RANKL-RANK signaling *in vitro* as well as *in vivo*, and was therefore an obvious

choice to be tested as an antiresorptive. Treatment with OPG in rodent models of OP was found to effectively lower the serum calcium levels in cancer-induced hypercalcemia (Boyle *et al.*, 2003), the development of an OPG therapy was scratched in favour of a fully human monoclonal antibody against RANKL (Martin and Seeman, 2007).

1.4.2.5 Denosumab

In an earlier chapter, we discussed in great detail the crucial role of the RANKL-RANK-OPG axis in the remodeling of bone. Ever since this axis was discovered, a number of strategies have been developed in an effort to manipulate this regulatory system in order to treat bone diseases. The approach that has thus far had the most success has been the development of denosumab - a monoclonal antibody to RANKL. Denosumab is administered intracutaneously, and has shown the potential to substantially reduce the number of osteoclasts upon dosing. As of May 2009, the drug has not yet hit the market, but is expected to do so soon. One potential side effect is the accumulation of microcracks in the bone due an excessive halting of resorption, resulting in brittle bone. As opposed to bisphosphonates, which stay in system for several years, denosumab is completely cleared from the body and as a result is not as likely to cause this problem. The main concern with denosumab, however, is that it could disrupt the global actions of the RANKL-RANK signaling pathway (Whyte, 2006). As described in earlier, the RANKL-RANK signaling system functions within in the immune system, and so shutting this down may result in immune dysfunction.

1.4.2.6 Calcitonin

Calcitonin, as described in great detail in an earlier chapter, is a very small peptide hormone (3.5kDa) that plays an important role in the regulation of calcium homeostasis in the body. It is a potent inhibitor of osteoclastogenesis in the body, and is therefore used as a therapy for postmenopausal osteoporosis. Salmon calcitonin, the most potent and tolerable of all other calcitonins, is commonly administered in the form of a nasal spray. In 1994, nasal salmon calcitonin efficacy was testing by the Prevent Reoccurrence of Osteoporotic Fractures, or PROOF trials. The results were released in 2000 by Chestnut *et al.*, and along with the results of previous studies led to the suggestion that nasal salmon calcitonin has only a small effect on bone density (Cummings and Chapurlat, 2000). So while nasal sCT treatment has been shown to mildly reduce the risk of fractures, it is minimally effective primarily as a result of issues of bioavailability (Chestnut *et al.*, 2000). Zaidi and Moonga suggest that this is to be expected as highly charged peptides like calcitonin are unlikely to cross the mucosal membranes effectively in the absence of a delivery vehicle. In spite of these issues with nasal salmon calcitonin, it is still widely used due to its claimed effect on bone mineral density, ease of use and lack of side effects (Coleman *et al.*, 2002). As a result of the short half-life of sCT (15-20min), the parenteral route requires frequent administrations in order to maintain the peptide's pharmacological effects (Huwyer *et al.*, 1979). Therefore, the patient would have to be subjected to daily injections and this would result in poor patient compliance. The oral route is therefore the preferred route, but the absolute bioavailability of salmon calcitonin administered in this way is extremely low. Studies have shown that sCT has very low intrinsic permeability across the intestinal membranes and undergo extensive proteolytic degradation in the

gastrointestinal lumen. In addition to this, peptide absorption is hindered by physical adsorption in the gut lumen (Antonin *et al.*, 1992). According to animal and clinical studies by Hastwell *et al.* in 1992, the bioavailability following colonic administration of human CT in rats have been found to in the order of 0.2% to 0.9%. In human studies, the bioavailability of hCT was found to lie between 0.05% and 2.7% after intracolonic administration (Antonin *et al.*, 1992; Beglinger *et al.*, 1992). A few of these downfalls of the oral administration of sCT have been overcome by the conjugation of polyethylene glycol (PEG). This conjugate was shown to achieve a sustained hypocalcemic effect, is a little more stable in the colon and less susceptible to adsorption (Mansoor *et al.*, 2005). Most recently, Chang and Lim (2009) have designed a non-ionic lipid conjugate of sCT (Lipeo-sCT) which has the same effectiveness as sCT in lowering the serum calcium levels in rodents (Cheng and Lim, 2008), and its bioactivity upon parenteral injection is seen to be extended by at least 6hrs when compared to a traditional parenteral sCT injection.

While it is evident that researchers are working on trying to enhance the bioavailability of sCT by increasing its absorbiton to and half-life in the serum, there is no evidence of any studies trying to increase bioavailability of sCT by targeting it directly to its receptors on osteoclasts.

1.4.2.7 Other Drugs Currently in Development

a. Cathepsin-K inhibitors

As described in an earlier chapter, cathepsin K is a cysteine protease that is highly expressed in osteoclasts. Cathepsin K is integral to the breakdown of the organic phase of bone during the process of bone resorption, and therefore inhibiting this enzyme would theoretically reduce the damage

caused by bone resorption. However, cathepsin K is also found in a number of other tissues such as skin cells, and skin-derived cells, and therefore an inhibition of its activity can potentially result in severe side effects. It has also been shown that the knocking out of cathepsin K in mice results in a higher predisposition to bleomycin-induced lung fibrosis (Buhling et al., 2004). For example, the cathepsin K inhibitor balicatib was discontinued after phase 2 trials due to the development of skin and respiratory side effects. Currently, the one cathepsin K inhibitor that has successfully completed phase 2 clinical trials without any obvious safety issues is Odanacatib. Odanacatib has been found to have an efficacy that is comparable to the potent bisphosphonates (Bone et al., 2007). Odanacatib is still in development, and appears to have the potential to be an effective antiresorptive therapy some day.

b. ATPase and Chloride Channel inhibitors

As described in an earlier section, one of the essential components of bone resorption by osteoclasts is the acidification of the resorption lacuna in order to dissolve the mineral layer of bone. The pH in the resorption pit is maintained by a vacuolar ATPase pump that actively transports protons into the lacuna. Therefore, inhibition of H^+-ATPase would, in theory, block bone resorption from taking place. Inhibitors of this enzyme have been tested as anti-resorptives, and one relatively osteoclast-specific H+-ATPase inhibitor has been shown to inhibit bone loss in rat OP models (Visentin *et al.*, 2000). In order to maintain electroneutrality during the course of acidification, it is essential for the transport of chloride ions into the resorption lacuna via chloride channels. As described by Martin and Seeman (2007), the absence of chloride channels will result in the hyperpolarization of the membrane and subsequently

the inhibition of further resorption. Studies by de Vernejoul *et al.* (2001) showed that mutations in the CLC7 gene are responsible for an osteopetrotic phenotype, and as a result, Schaller *et al.* (2004) developed an orally delivered inhibitor of the CLCN7 chloride channel. This therapy was shown to inhibit bone loss in a rat OP model without inhibiting bone formation.

c. Disintegrins (Echistatin, Kistrin)

The crucial role played by the integrin $\alpha v\beta 3$ receptor in osteoclast activity has been the target of antiresorptive called disintegrins. Disintegrins like echistatin and kistrin act by binding with high affinity to the $\alpha v\beta 3$ integrin, inhibiting bone resorption (Martin and Seeman, 2007). As described by Coleman et al. (2004), small peptides that mimic the RGD peptide sequence that is recognized by the $\alpha v\beta 3$ receptor were also shown to have similar effects on bone resorption.

1.5 TARGETED DRUG DELIVERY

The targeting of a drug molecule to a particular site of action results in reducing the amount of drug being circulated to other organs and tissues. As a result, the chances of any side-effects on other organs or tissues is reduced considerably while there is an increase in the bioavailability of the drug at the site of action, causing a tremendous increase its therapeutic potential (Kidane and Bhatt, 2005). In this chapter, the use of antibodies and related conjugates as delivery mechanisms will be discussed.

1.5.1 Antibodies as Drug Carriers

The use of antibodies as carrier vehicles for other drugs ensures the selective delivery of the payload directly to the diseased or disease-causing cells. The drugs that benefit from this form of delivery are those that either have high system toxicity or low target tissue absorption.

Antibody-based targeted delivery requires the therapeutic drug to be conjugated to the carrier antibody in a covalent fashion. This is so as to make sure that the linkage is stable enough to keep the drug attached to the carrier antibody until the conjugate reaches the target tissues/cells. If cleavage of the drug from the antibody is required at the site of targeting, the linkage between the carrier antibody and the drug should be selected so that the mechanism of cleavage depends on the microenviroment of the target site. Once they arrive at the site of action, the conjugates can function either intracellularly or extracellularly.

Intracellularly Acting Conjugates

As described by Shen and Louie in 1999, intracellularly acting conjugates are generally designed for drugs that target DNA or enzymes within the cells. These conjugates need to be internalized, and this is usually via endocytosis. Upon uptake by vesicles, the conjugate is processed in the endosomes and lysosomes, and proteolytic enzymes in the lysosomes degrade the antibody. Assuming the drug molecule survives the processing in the lysosomes, it can start acting on its target within cell. For example, methotrexate (MTX) and daunorubicin (DNR) are two drugs that can be bound to antibodies and get internalized by the cell. Following lysosomal processing, they emerge as MTX-

lysine and DNR-glutamate respectively. While MTX-lysine is active, DNR-glutamate is not.

Extracellularly Acting Conjugates

As stated by Shen and Louie (1999), any antibody-conjugates that can exert their activity extraceullarly can act in two main ways: either they act on the targets in the cytoplasm, or they are released from the antibody onto the surface of their target cells and generate a depot effect. The limiting factors of this strategy are the number of drug molecules that can be delivered per antibody, and the number of drug molecules that act on each antigenic epitope on the cell. The rate of release from the antibody can also be a limiting factor. One strategy to get around this to generate active drug molecules at the target site using an enzyme antibody conjugate that converts an inactive prodrug into an active drug. For example, etoposide phosphate is delivered and activated at its site of action by a phosphatase-antitumor antibody conjugate. These potential drawbacks, however, are a non-issue if the therapeutic being delivered has a potent effect on its target cells.

Safety and Efficacy of Antibodies in Therapeutics

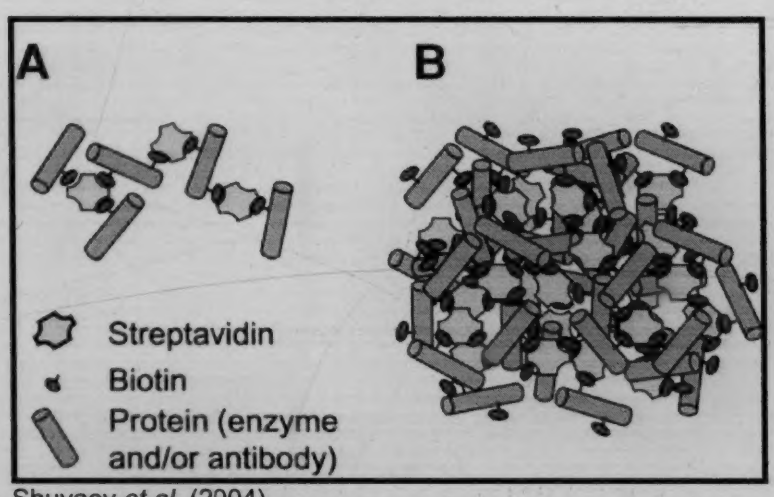
In the last decade, the number of antibodies and antibody-conjugates being used in therapeutic applications to treat human disease has steadily risen, with several more in the pipeline. As antibodies are different from conventional drugs, they need to satisfy different safety and efficacy criteria. For example, the antibodies used in therapy are generally isolated from human serum, and therefore must be screened very carefully in the same way that other blood products are screened. Also, the increasingly popular monoclonal antibodies are

produced by hybridoma technology and therefore need to be carefully screened for any sort of contamination. Another safety requirement for antibodies in therapeutic strategies is that they do not cross-react with other tissues or cells in a non-specific fashion.

1.5.2 Streptavidin-Antibody Conjugates in Drug Delivery

As described by Shuvaev *et al.* (2004), one alternative to drug delivery via conjugation to an antibody, is to use the streptavidin-biotin system. Streptavidin and biotin are two proteins that have an extremely high affinity for one another, and this relationship has been the focus of studies that are looking to exploit the system for purposes of drug delivery. The extraordinary interaction between streptavidin and biotin has a K_d of 10^{-15}M, and is a noncovalent interaction. This extremely specific and nearly irreversible interaction is widely used in biology as well as in medicine (Wilchek and Bayer, 1990). The covalent linking of a protein with biotin results in the formation of a biotinylated protein that will now bind to streptavidin and form a protein-streptavidin conjugate. According to Shvaev *et al.* (2004), these conjugates can be characterized into two main categories depending on the level of biotinylation: oligomeric and polymeric conjugates. Oligomeric conjugates are used commonly in labeling techniques, and occur when the protein contains less than two biotins per molecule. When a protein has an average of two of more biotins per molecule, the structure formed is a polymer, as shown in Fig. 1.3, Shuvaev *et al.* in 2004 constructed a novel conjugate that was intended to deliver the therapeutic molecule catalase specifically to cell adhesion molecules (PECAM) on epithelial cells lining the luminal surface of the vasculature. Their design involved the biotinylation of

catalase as an anti-PECAM antibody, followed by their conjugation to a streptavidin molecule. The result was the formation of a conjugate that targeted catalase to PECAM on epithelial cells using the streptavidin-biotin system. This particular strategy of targeted delivery promises to be a very important laboratory tool, especially in the designing of 'proof-of-principle' conjugates that can be tested *in vitro*.



Shuvaev *et al.* (2004)

Fig. 1.3: Depiction of the two types of protein conjugates that are formed with the streptavidin-biotin system. Proteins with less than two biotin/molecule (oligermeric conjugates) are shown in A, and proteins with two or more biotin/molecule (polymeric conjugates) are shown in B.

1.6 *IN VITRO* SYSTEM TO STUDY OSTEOCLASTS

The first breakthroughs in studying the mechanisms involved in osteoclast differentiation and activation came about when co-cultures of bone marrow cells with stromal cells resulted in the generaton of osteoclasts (Takahashi *et al.*, 1988). For several years after this finding, co-culture systems have been used to study the differentiation and maturation process of osteoclasts (Suda *et al.*,

1999), but this system is not always ideal as it is a mixture of two types of cells. As described by Meiyanto *et al.* (2001), the co-culture is not suitable for studies who aim to analyze the details of osteoclastogenesis and osteoclast maturation. Hsu *et al.* (1999) were the first to report that the mouse leukemic monocyte macrophage derived cell line RAW 264.7 (Ralph *et al.*, 1977) could be induced to form mature osteoclasts in the absence of stromal cells by dosing them with purified RANKL (Hsu *et al.*, 1999) and M-CSF (Yoshida *et al.*, 1990). As described by Wittrant *et al.* (2003), before the RAW 264.7 cell culture system was developed, researchers had to purify mature osteoclasts from total rabbit bone cells. Even though the purity was deemed to be approximately 95%, the presence of even a small poportion of stromal cells will result in the the uncontrolled activation of osteoclastogenesis. Therefore, the use of a murine monocytic cell line RAW 264.7 gives researchers the opportunity to test the effects of RANKL in a controlled manner. In addition RAW 264.7 cells are readily accessible and constitute a homogenous cell culture system. As described by Collin-Osdoby *et al.* (2003) a large number of osteoclasts can be generated from RAW 264.7 cells and they have been shown to have high resorptive capabilities. As a result, the RAW 264.7 cell culture system is currently the preferred method of culturing and studying osteoclasts.

CHAPTER 2

OVERVIEW

AND

HYPOTHESES

2.0 OVERVIEW AND HYPOTHESIS

Osteoporosis is a serious and prevalent bone disease that results in the loss of bone mass through osteoclast-mediated bone resorption. Current therapies have either been shown to be associated with severe side-effects after long-term usage (SERMs, Bisphosphonates), or have issues of bioavailability that render them largely ineffective (nasal salmon-Calcitonin). Newer therapies such as denosumab are not yet fully established, and so the potential long-term efficacy and side-effects remain to be confirmed.

Of the above mentioned therapies, salmon calcitonin (sCT) is the therapy associated with the least side-effects. The main reason for sCT not currently being a frontline therapy is poor bioavailability when administered either nasally or parenterally. Studies have shown that a majority of the administered dose of sCT never reaches the bone microenvironment, and so the effect is minimized considerably. As a result, we believe that targeting sCT directly to osteoclasts in the bone microenvironment will circumvent the issue of bioavailability to a great degree. We propose that sCT should be delivered to osteoclasts using a system that targets the sCT payload directly to these cells. The target we propose to use is the surface receptor RANK (receptor activator of NF-κB). Targeting RANK with sCT will result in the delivery of sCT to all cells that display RANK on their surface. While this will include cells other than osteoclasts, sCT does not have any effect on any RANK-displaying cells other than osteoclasts.

Rationale for a "sCT- anti-RANK" Conjugate

As was discussed in earlier chapters, RANK and CT/R both exist on numerous different cell types. For example, RANK can be found on dendritic

cells and is an essential part of T cell – dendritic cell communication. Therefore it is fair to speculate that dosing with a conjugate that targets RANK will result in the targeting of all of these other cell types. This is where the importance of CT as the therapeutic molecule is truly recognized. CTRs, while they are found on several other cell types, are predominatly found on one type of cell that also contains RANK on its surface. These cells are the bone resorbing osteoclasts. Therefore we propose that while a RANK-targeting conjugate will bring CT in close proximity to other cell types, there will be no effect on these cell types unless they express CTRs. As mentioned above, osteoclasts contain both, and therefore will be the major cell type to be affected by the delivery of CT in their microenvironment.

Therefore, our general hypothesis is: **The osteoclast surface receptor RANK can be used as a target to deliver therapeutic anti-resorptive peptides such as calcitonin directly to osteoclasts.** In order to test our general hypothesis, we will explore three sub-hypotheses that have been outlined below along with the specific aims associated with each sub-hypothesis.

Sub-Hypothesis # 1: Osteoclast precursors (RAW 264.7 cells) can be targeted using antibodies to the receptor RANK, without any unwanted stimulation of osteoclastogenesis.

Specific Aim 1: To confirm the dose-dependent targeting of Anti-RANK molecules to the receptor RANK on precursor RAW 264.7 cells.

We will use monoclonal antibodies (MAbs) that target RANK, and detect their localization on monolayers of RAW 264.7 cells using an

enzyme-conjugated secondary antibody and an ELISA. The colorimetric intensity of the ELISA will be quantified using an automated plate reader.

Specific Aim 2: To use molecular techniques to confirm that osteoclastogenesis has not been triggered following treatment of RAW 264.7 cells with Anti-RANK.

Monolayers of RAW 264.7 cells will be treated with either anti-RANK MAb or RANKL, and be harvested at 24 hr after dosing for reverse-transcription polymerase chain reaction (RT-PCR). Expression of the downstream gene NFATc1 will be used to indicate the triggering of the RANK receptor.

Sub-Hypothesis # 2: Dosing mature osteoclasts *in vitro* with an anti-Calcitonin antibody conjugated to calcitonin [CT- (anti-CT)] should result in the delivery the calcitonin to its receptors on osteoclasts.

Specific Aim 3: To determine whether the dosing of mature osteoclasts with a CT-(Anti-CT) immunoconjugate results in the delivery of CT to its high-affinity receptors on osteoclasts.

Osteoclasts will be generated from RAW 264.7 cells and seeded onto a 24-well plate. This monolayer of osteoclasts will then be treated with the CT- (anti-CT) molecule, which formed by the conjugation of CT to polyclonal anti-CT molecules. The treatment group wells will be subjected to an ELISA using a secondary antibody that recognizes CT, and the colorimetric change will then be analyzed qualitatively.

Sepcific Aim 4: *To determine whether upon delivery of CT to its high-affinity receptor on osteoclasts, the antigen will detach itself from the complex and cause the anti-CT antibodies to be washed away.*

Following treatment of an osteoclast monolayer with the CT-(anti-CT) conjugate, an ELISA is performed using a secondary antibody that recognizes the polyclonal anti-CT molecules. The colorimetric change will be analyzed qualitatively.

Sub-Hypothesis # 3: The conjugation of biotinylated Calcitonin and biotinylated Anti-RANK to streptavidin will result in the formation of a conjugate CT-ST- anti-RANK that will target osteoclast receptors *in vitro*, thereby introducing CT into the microenvironment.

Specific Aim 5: *To determine if the delivery of the CT-ST-anti-RANK conjugate onto immobilized RANK results in the Anti-RANK arm targeting RANK, thereby delivering CT into the osteoclast-mimicking microenvironment.*

Recombinant soluble human RANK will be immobilized onto the wells of a 24-well plate, and treated with a CT-ST-anti-RANK conjugate that will be formed by conjugating equimolar amounts of CT and anti-RANK to ST in a two-step conjugation. Then an ELISA will be performed using a secondary antibody that will detect the presence of CT in the osteoclast-like environment. A plate reader will be used to measure the colorimetric change.

CHAPTER 3

MATERIALS

AND

METHODS

3.0 MATERIALS AND METHODS

3.1 Growing, Propagating, and Stocking of RAW 264.7 cells

RAW 264.7 cells were purchased from ATCC (VA, USA) and thawed first on dry ice (20min) and then in water (1 minute). GIBCO High Glucose 1X Dulbecco's Modified Eagle Medium (DMEM) was then added to the thawed out RAW 264.7 cells, and spun down at room temperature (RT) for 7min at 485g in a centrifuge. The GIBCO DMEM was purchased from Invitrogen (CA, USA) and contained 4.5 g/L D-Glucose, L-Glutamine, and 110 mg/L Sodium Pyruvate. The DMEM then had 5% FBS and 1% Penicillin-Streptomycin (10000 U/ mL; Invitrogen) added to it, resulting in RAW 264.7 cell culture medium. The pellet recovered following the centrifuging was then resuspended in 25mL of RAW 264.7 culture media (DMEM, +FBS, +Penicillin-Streptomycin) and transferred to a CORNING 75 cm^2 Canted Neck flask (Corning International, NY, USA). The flask was then placed in a Thermo Fisher Scientific Water Jacketed CO_2 incubator (37°C, 5% CO_2) until the cells were confluent (3-5 days).

Following the achievement of confluence, the cells in the flask were scraped using a BD Falcon Cell Scraper (BD Biosciences, MA, USA), collected in a 50mL BD Falcon tube, and then spun down in the centrifuge (RT, 7min, 485g). The supernatant was decanted, and the pellet was resuspended in 10mL of media. The resuspended cells were then divided into two 75 cm^2 flasks, and a further 20mL of RAW 264.7 culture media was added to each flask. Media was also added to the parent flask, which was returned to the incubator to serve as backup. The cells in the two daughter flasks were labeled as Passage 1 (P1) and placed in the incubator for 48hrs, after which the process of dividing and

propagating the cell culture was repeated as described above, until the cells reached P8 (i.e., 8 propogations)

Upon reaching P8, the cells from the two P8 flasks were scraped, collected, and centrifuged (RT, 7min, 485g). The cells the first Falcon tube were then resuspended using 13.5mL FBS and 1.5mL of Dimethylsulfoxide (DMSO; Sigma-Aldrich, MO, USA), and this suspension was transferred to the second Falcon tube to resuspend the second pellet. The 15mL suspension was then aliquoted into cryovials (1mL/cryovial) and placed at -80°C in a slow-freeze cryovial box, and transferred to liquid nitrogen after 24hrs for long-term storage.

To propagate RAW 264.7 cells from the liquid nitrogen stock, the cryovial is placed on dry ice (20min), followed by ice (15min), and then room temperature to thaw out the RAW 264.7 cells in a controlled manner. The 1mL of stock is then added to 15mL of media, and centrifuged (RT, 7min, 485g). The supernatant is discarded, and the pellet is resuspended in 25mL of media and transferred to a $75cm^2$ flask and placed in the CO_2 incubator until the cells reach confluence.

3.2 Triggering Osteoclastogenesis in a 24-well Plate

To generate osteoclasts in a 24-well plate, RAW 264.7 cells are first scraped and collected from one $75cm^2$ flask, centrifuged, and resuspended in 25mL of media. The cells are then transferred to a 24-well plate (1mL of cell suspension/well) and placed in the CO_2 incubator overnight to allow the cells to attach to the surface. After 24hrs, the culture medium in the wells is replaced with media that has been spiked with MCSF (PeproTech, NJ, USA) and RANKL (PeproTech, NJ, USA) at concentrations of 25ng/mL. After 24hrs, the media from all the wells is aspirated and the wells are treated with HBSS for 1 minute. The HBSS is then aspirated and 250µL of TRIZOL is added to each well.

Following 30 seconds of swirling, the cells in each well are scraped using a cell scraper, and collected in microfuge tubes. The tubes are all vortexed, and allowed to sit at room temperature for 5 min after which they are vortexed again and placed in the -20°C (if RNA Extraction will be performed later) or 4°C (if RNA Extraction is to be performed the same day).

3.3 RNA Extraction and Reverse Transcription

The RNA from the RAW 264.7 cells was isolated using a QIAGEN RNeasy Mini Kit (QIAGEN Sciences, MD, USA). The procedure involves first separating nucleic acids from proteins using Chloroform:Isoamyl Alcohol (300µL/mL of TRIZOL), before treating the sample with DNase to degrade DNA. The sample is then applied onto a membrane that selectively binds RNA while allowing contaminants to be washed away. The RNA is then eluted using a proprietary elution buffer provided by QIAGEN Sciences. Following extraction, a small volume of RNA from each sample was treated with SYBRgreen II reagent (Molecular Probes, Oregon, USA) before being inserted into a Fluorimeter. The Fluoroskan Ascent program was then used to quantify the RNA in each sample using the wavelengths of excitation (460nm) and emission (535nm). Following determination of RNA concentration in each sample, appropriate dilutions were carried out in order to have up to 1µg of RNA in a 30µL volume for Reverse Transcription into cDNA. Reverse transcription of RNA to cDNA was carried out using the QIAGEN Omniscript RT Kit (QIAGEN Sciences, Maryland, USA). Briefly, 30µL of each sample was treated with 10µL of a master mix that was made up by mixing the provided RT buffer, dNTPs, random primers (hexamers, $pd(N)_6$), RNAGuard, and Reverse Transcriptase enzyme as described by QIAGEN. Upon treating all the samples with the 10µL of the master mix, the

tubes are incubated in a 37°C water bath for 1 hour. After the incubation, each sample was diluted by adding 110µL of ultra-pure H_2O, spun down for 15 seconds, and placed at -30°C for future use.

3.4 PCR amplification of NFATc1

In order to amplify NFATc1 from the cDNA samples, a QIAGEN Taq DNA Polymerase Kit was used. First, amplification of the housekeeping gene GAPDH by the Polymerase Chain Reaction allowed for the equalization of the amount of DNA in the samples. The forward and reverse GAPDH primers used were: 5'-GGTCGGTGTGAACGGATTTG-3' and 5'-GTGAGCCCCAGCCTTCTCCAT-3' (Kruse *et al.*, 2002). Upon completion of 30 cycles of PCR (94°C, 54°C, 72°C), the samples were loaded onto a 2% Agarose gel and run for 1hr at 100 V. The gel was then soaked in ethidium bromide for 30 minutes, and then soaked in water for 30 minutes. Then the gel was placed under UV light in the Alpha Imager to record the results. The samples were then equalized, i.e., analyzed to ensure that there was equal expression of GAP, therefore suggesting equal amounts of genomic DNA in all samples. The NFATc1 gene was then amplified by PCR (40 cycles; 94°C, 45°C, 72°C) using the primers: 5'-GGTAACTCTGTCTTTCTAACCTTAAGCTC-3' and 5'-GTGATGACCCCAGCATGCACCAGTCACAG-3' (Asagiri *et al.*, 2005). The PCR products were then run on a 2% agarose gel, and visualized using ethidium bromide staining as described above.

3.5 Generation and Visualization of Osteoclasts

3.5.1 Determining the Ideal Seeding Density for Osteoclast Formation

RAW 264.7 cells were scraped from a flask, spun down in a centrifuge, and resuspended in 15mL of media. The number of cells in the suspension was measured by loading 10µL of the suspension onto a Bright-Line Hemacytometer (Hausser Scientific, PA, USA). Following the determination of the concentration of the culture suspension, serial dilutions were conducted resulting in 2mL each of $2x10^6$, $1x10^6$, $1x10^5$, and $1x10^4$ cells/mL. The cells were seeded onto wells of 24-well plate such that each concentration of cells was seeded in quadruplicate (4 wells, at 1mL/well). In order to grow osteoclasts, dosing with peptide factors MCSF and RANKL (PeproTech, NJ, USA) at 25ng/mL is repeated every 48hrs for 7 days. On Day 8, osteoclasts can be visualized by staining for Tartrate-resistant acid phosphatase (TRAP).

3.5.2 Generating osteoclasts in a 25 cm^2 flask

RAW 264.7 cells were scraped from a flask, spun down in a centrifuge, and resuspended in 15mL of media. The number of cells in the suspension was measured by loading 10µL of the suspension onto a hemacytometer. Following the determination of concentration of cells in the suspension, 25mL of cells at $1x10^4$ cells/mL was made up and used to seed all wells of a 24-well plate (1mL/well). Following dosing with MCSF (PeproTech, NJ, USA) and $RANKL_W$ (kindly provided by Dr.Suresh) every 48hrs over the course of 7 days, osteoclasts were scraped using a cell scraper. The osteoclasts were then pooled and transferred into two 25 cm^2 flasks. Dosing with MCSF and $RANKL_W$ was carried out every 48hrs over the course of 6 days in one flask, while the other flask was a treated as a control and therefore only treated with cell culture media (no peptide factors). Following 6 days of growth, osteoclasts in the first flask were counted under a microscope after dividing the flask into 12 fields of view.

Then, the cells in the flask were scraped and 10µL of the suspension was analyzed using a Bright-Line Hemacytometer to determine the concentration of undifferentiated RAW 264.7 cells. We were then able to calculate the number of osteoclasts per mL of RAW 264.7 cells.

3.5.3 Visualizing Osteoclasts using the TRAP-activity Assay

TRAP staining was carried out using the Leukocyte Acid Phosphatase (TRAP) Kit from Sigma-Aldrich (St.Louis, MO, USA). First, 200µL of Citrate concentrate provided in the kit was added to 1.8mL of dH_2O in a 15mL Falcon tube, giving 2mL of Citrate solution (0.38mol/L, pH 5.4). This solution was then transferred into a 50mL Falcon tube containing 3 mL of Acetone, resulting in 5mL of Citrate-Acetone solution. Also, two Falcon tubes filled with 46mL and 48mL of dH_2O respectively were placed in a 37°C water bath for use later in the process. The principle behind staining for TRAP activity involves the use of napthol AS-BI phosphates in conjunction with diazonium salts for the detection of acid phosphatase, and it was first used in leukocytes by Goldberg and Barka (1962). According to Sigma-Aldrich, the Acid Phosphatase Leukocyte kit used in this study introduces tartrate along with a napthol AS-BI phosphate, which upon contact with TRAP is enzymatically hydrolyzed resulting in the release of napthol AS-BI (7-Bromo-3-hydroxy-2-naphtho-o-anisidine). Napthol AS-BI then couples with the stable diazonium fast garnet GBC salt that is formed in the presence of sodium nitrite in an acidic medium, resulting in the formation of insoluble maroon deposits at sites of acid phosphatase activity. Cells containing tartrate-sensitive acid phosphatase are devoid of enzymatic activity due to the introduction of tartrate into the system in the first step, and therefore do no show any maroon deposits in the cells. Following the staining for TRAP activity, hematoxylin is

added to the system in order to stain all TRAP-negative cells a dark blue/black colour. This enables easy visual identification of osteoclasts against a background of undifferentiated RAW 264.7 cells The media from each well of the 24-well plate is aspirated, and replaced with warm HBSS (1mL/well). After 1 minute, the HBSS is aspirated and 270µL of the Citrate-Acetone solution is added to each well. The Citrate-Acetone is aspirated after 45 seconds, and 1mL of dH_2O is added to each well and the plate is placed back in the CO_2 incubator for 15 minutes. During the 15 minute treatment with dH_2O, the two Falcon tubes are removed from the water bath and labeled as Solutions A (46mL) and B (48mL). Add 2mL of the provided Acetate solution (to give 2.5mol/L, pH 5.2) and Naphthol AS-BI Phosphoric Acid solution (12.5 mg/mL) to each tube. To Solution A, add 2mL of Tartrate solution (to give 0.67/mol/L, pH 5.2). Then, add the powder from one Fast Garret GBC Salt capsule to each of Solutions A and B. Swirl the tubes for 30 seconds, and then filter through a No.1 Whatman filter paper into two new Falcon tubes. Place the filtered Solutions A and B in a water bath (37°C) for 10min. Aspirate the dH_2O from the wells, then add 1mL of Solution A to half the wells and 1mL of Solution B to the other half. Place the plate in the 37°C CO_2 incubator for 1 hour. After the incubation, aspirate the solutions from the wells, and add 1mL of dH_2O to each well. Aspirate after 30 seconds, and add 1 mL of Hematoxylin (1 g/L, pH 3.3 at 25°C) from the kit to each well. Let sit for 5 min, then aspirate and add 1mL of dH_2O to each well set aside for 3 min. Then, aspirate the dH_2O and air-dry in a biosafety hood for 15-30min or until there is no visible wetness. Osteoclasts can then be detected by viewing wells treated with Solution B under a microscope. Using a camera attached to the microscope, the results were recorded as images for later analysis.

3.6 Resorption assay

Effect of drug intervention on resorption by osteoclasts

Osteoclasts were generated in a $25cm^2$ flask by dosing RAW 264.7 cells seeded at $1x10^4$ cells/mL with M-CSF and RANKL as described earlier. Following 6 days of growth in the flask, osteoclasts were scraped and counted under a microscope before being seeded onto six wells of a 16-well BD Biosciences Osteologic Slide (BD Biosciences, MA, USA). Three other wells involved in the treatment were left empty to serve as a negative control. All nine wells were treated with M-CSF and RANKL for three days, after which three out of the six osteoclast-seeded wells were treated with Risedronate (50μM) everyday over the course of 3 days while the other three osteoclast-seeded wells and the negative control wells (i.e., no-cell control) were dosed only with M-CSF and RANKL. After 3 days, high magnification pictures were taken using a camera connected to a microscope (courtesy of Dr. El-Bialy) before the structure forming the wells of the slide was removed as per instructions provided by BD Biosciences. The slide was then placed in a petri-dish and washed with MQ H2O. The slide was then soaked in bleach for 10min before being washed 3X with MQ H2O. Next, the slide was soaked in 0.1% Toluidine solution for 5 minutes, and subsequently washed 3X with MQ H2O. Following air-drying, the slide was viewed under the microscope and high magnification images of the osteologic (Calcium Phosphate) material remaining on the slide were recorded. In order to quantify the resorptive effect of osteoclasts, the images were analyzed using the java-based image-processing program ImageJ (NIH, USA).

3.6 Enzyme Linked Immunosorbent Assays against various antigens

3.6.1 Binding of a monoclonal antibody to RANK on RAW 264.7 cells

RAW 264.7 cells were seeded onto wells of a 96-well plate and allowed to attach overnight. The cells were washed three times with 0.01M PBS, and then blocked with a 2% BSA-PBS solution for 1.5 hours at 37°C. Following three more washes with 0.01M PBS, mouse monoclonal antibody to RANK (Biocarta, San Diego, USA) was spiked into wells of each treatment group at different concentrations (1µg/mL, 5 µg/mL, and 10µg/mL). After 1.5 hours of incubation at 37°C, the wells were washed three times with 0.01M PBS and Goat Anti-Mouse IgG-HRPO (provided kindly by Dr.Suresh) was added to each well. The plate was incubated at 37°C for 45 minutes before TMB (provided by Dr. Suresh), which is a substrate for HRPO, was added to each well. The colorimetric change was measured at an absorbance of 650nm using a BioTek EL 808 automated plate reader (BioTek Instruments Inc., Winooski, VT)

3.6.2 Delivery of Calcitonin to Osteoclasts by an Anti-CT Carrier Antibody

RAW 264.7 cells were seeded overnight onto wells of a 24-well plate and dosed with MCSF (25ng/mL) and RANKL (25ng/mL) the following day. Dosing with peptide factors was carried out every 48hrs over the course of 7 days. In the meantime, human Calcitonin was purchased in a biotinylated form (AnaSpec, San Jose, USA) and reconstituted first in water and then PBS as per the recommendations. Human Calcitonin was then complexed, in equimolar quantities, with Rabbit polyclonal antibodies to human Calcitonin (Biocare Medical, CA, USA). Upon formation of osteoclasts, the wells were blocked with 1% BSA-PBS for 1.5hrs and washed three times with 0.01M PBS before being treated with the Antibody-Calcitonin complex. After 1.5hrs, the wells were

washed three times with 1:1/10 PBS. Half the wells were then treated with Goat Anti-Rabbit antibody-HRP(I) to detect the rabbit carrier antibody while the other wells were treated with Streptavidin-HRP(I) to detect free Calicheamicin. Pictures were then taken of the wells using a camera and the results were analyzed qualitatively.

3.6.3 Binding of the CT-57 (Anti-Ki67) conjugate to immobilized Ki67

Soluble human Ki67 (10ug/mL) was added to 12 wells of a 96-well High Protein-Binding plate to a final concentration of 1ug/well, and allowed to attach overnight at 4°C. The same coating technique was carried out for 12 more wells. The wells were washed three times with 1:1/10 PBS, and then blocked with a 2% BSA-PBS solution for 1 hour at 37°C. Following three more washes with 1:1/10 PBS, the CT-57 (Anti-Ki67) conjugate was added at two concentrations (1/100 and 1/500) in triplicate to Ki67-coated and BSA-coated wells. Streptavidin (1 (10mg/mL) and PBS were also each added in triplicate to both the Ki67-, coated and BSA-coated wells. After 2 hours of incubation at 37°C, the wells were washed three times with 1:1/10 PBS and Rabbit anti-human Calicheamicin (Biocare Medical, CA, USA) was added to all the wells. The plate was incubated at 37°C for 1hr, washed, and treated with Goat Anti-Rabbit antibody-HRP(I). After 40 minutes of incubation at 37°C, TMB substrate was added to each well. The colorimetric change was measured at an absorbance of 650nm using a BioTek EL 808 automated plate reader.

3.7 Formation of CT-Streptavidin-(Anti-RANK) Conjugate

The formation of the CT-Streptavidin-(Anti-RANK) conjugate was carried out by mixing biotinylated- human CT (Anaspec) and biotinylated murine Anti-human soluble RANK (Peprotech), or simply Anti-hRANK, in equimolar amounts such that the mixture contained 2×10^{-10} moles of each reactant. After mixing them well, 1×10^{-10} moles of streptavidin (Invitrogen) was added to the mixture of biotinylated hCT and Anti-hRANK. The three reactants were mixed well and placed at room temperature for 2 hours. Following the incubation at RT, the conjugation mixture was placed at 4°C overnight. The following day, a 1/100 and 1/500 dilution of the conjugate mixture was analyzed using an enzyme-linked immunosorbent assay that was carried out on wells containing immobilized human soluble RANK (Peprotech, NJ, USA) as described in 3.7.3 above.

3.8 Statistical Tests

All results on figures are presented as the mean $\pm$ standard error of the sample group. The statistics for all values seen in figures and tables were computed using the Microsoft EXCEL program. Unpaired two-tail t-tests were carried out, and significance between groups was reported if the P-value was below 0.05.

CHAPTER 4

RESULTS

4.1 Triggering osteoclastogenesis in RAW 264.7 cells

Following a 24hr treatment of RAW 264.7 cells in triplicate with M-CSF (25ng/mL) and varying concentrations of RANKL (0ng/mL, 5ng/mL, 10ng/mL), the cells were harvested using TRIZOL. The cells from the three wells of each treatment were pooled and the RNA was extracted, mRNA was transcribed to cDNA, and PCR was used to amplify the NFATc1 gene as has been described earlier. The results (Fig. 4.1) were acquired by visualization under UV light using the Alpha Imager. The DNA from RAW 264.7 cells that were treated with M-CSF (25ng/mL) and 0ng/mL of RANKL showed a very light band at ~400bp, while DNA from RAW 264.7 cells treated with M-CSF (25ngmL) and 5ng/mL of RANKL showed a brighter band at the same position. The brightest band seen was from the DNA of RAW 264.7 cells that were treated with M-CSF (25ng/mL) and 10ng/mL of RANKL.

4.2 Generation and Visualization of Osteoclasts *in vitro*

RAW 264.7 cells were seeded in duplicate at $2x10^6$, $1x10^6$, $1x10^5$, and $1x10^4$ cells/mL onto wells of a 24-well plate, The cells were then dosed with M-CSF and RANKL every 48hrs over the course of 7 days. Following TRAP staining, images of a mature osteoclast was recorded using regular as well as phase-contrast microscopy (Fig.4.2). The number of osteoclasts generated from each concentration of RAW 264.7 cells was recorded using a microscope, and the size of the osteoclasts was determined qualitatively: osteoclasts that had less than four nuclei were labeled 'medium-sized' whereas osteoclasts with over 4 nuclei were labeled as being 'very large'. There were no mature osteoclasts seen in wells that were seeded with RAW 264.7 cells at $2x10^6$ and $1x10^6$ cells/mL, and an average of 19 medium-sized osteoclasts were seen in the two wells of RAW

264.7 cells that were seeded at $1x10^5$ cells/mL (Fig.4.3). No large osteoclasts were seen in either of these three treatments. In wells that had RAW 264.7 cells seeded at $1x10^4$ cells/mL, there was an average of 27.5 medium-sized osteoclasts and also an average of 9 very large osteoclasts (not shown in data).

4.3 Testing the efficacy of Osteoclasts using an *in vitro* resorption assay

Mature osteoclasts were grown in a flask and seeded onto 6 wells of 16-well slide coated with osteologic material (calcium phosphate). Of the six osteoclast-seeded wells, the osteoclasts in three were dosed with Risedronate after 3 days while the other three osteoclast-seeded wells were allowed to continue growing in the presence of M-CSF and RANKL for another 4 days. The three wells that contained no cells were also treated with M-CSF and RANKL for another 4 days. At the end of the experimental period, the osteoclasts on the osteologic material were visually analyzed under a microscope (Fig. 4.4 A). The osteoclasts were then washed off using bleach and the osteologic material was stained with toluidine blue that allowed for visualization of osteoclast-formed pits following the removal of osteoclasts (Fig. 4.4 B). When analyzed visually, the osteoclast-seeded wells that were treated with Risedronate showed a high level of calcium phosphate conservation when compared to the untreated osteoclast wells, but the level of conservation appeared in Risedronate-treated osteoclast wells seemed lower than that seen in the negative control (Fig. 4.5). Using the Image J software, the full colour images were converted ino binary images by applying a threshold. The number of black and white pixels was then measured using Image J software, and it was determined that the percentage conservation of calcium phosphate in Risedronate-treated osteoclast wells was significantly higher than that seen with the untreated osteoclast wells (Fig. 4.6). The three

negative control wells, none of them seeded with any cells, showed a very high level of calcium phosphate. However, the difference between the amount of resorption seen in negative control and the Risedronate-treated osteoclasts was not significant.

4.4 Treatment of RAW 264.7 cells with the Anti-RANK antibody

4.4.1 Binding of Anti-RANK to RANK on RAW 264.7 cells

RAW 264.7 cells were seeded onto 16 wells of a 96-well plate overnight and 12 of these wells were treated with 1μg, 5μg and 10μg of murine anti-RANK in triplicate the following day. The four other RAW 264.7-seeded wells were left untreated. An ELISA was then performed using goat anti-mouse IgG-HRPO. TMB substrate was then added to these wells and the colorimetric change was measured at 650nm using BioTek EL808 plate reader (Table 4.1). The colorimetric change was lowest in RAW 264.7 cells treated with BSA-PBS (NCON1). The colorimetric change in RAW 264.7 cells treated with 1μg of anti-RANK was slightly, yet significantly, higher than that seen in NCON1. There was a doubling in the colorimetric change in cells treated with 5μg of anti-RANK when compared to NCON1, and this change was deemed to be significant. Cells treated with 10μg of Anti-RANK showed a significant colorimetric change when compared to that seen in the negative control NCON1, and was 1.67 times higher than the colorimetric change seen in cells treated with 5μg of anti-RANK. The colorimetric change was also significantly different between the three anti-RANK treatments. A photograph was taken in order to allow for a visual qualitative analysis (Fig.4.7)

4.4.2 NFATc1 levels in RAW 264.7 cells after treatment with Anti-RANK

RAW264.7 cells were seeded onto 16 wells of a 24-well plate overnight and then then treated with 0μg (NCON1), 5μg, 10μg, and 20μg of anti-RANK for 24 hours before subsequently being harvested. Following RNA extraction and conversion to cDNA, a PCR was performed to amplify the NFATc1 gene. Following staining with ethidium bromide, the DNA bands were visualized using the Alpha Imager (Fig.4.8). The band at ~400bp was very weak, and of very similar intensity in all of the treatments.

4.5 Dosing mature osteoclasts with bCT-(Anti-CT) immunoconjugate

Mature osteoclasts were generated and seeded onto 10 wells of a 24-well plate. Of these, 6 wells were treated with a conjugate that was created by reacting biotinylated human calcitonin to a polyclonal antibody to human calcitonin. The other 4 wells were treated only with PBS. Then, an ELISA was performed on 3 conjugate-treated osteoclast wells and 2 PBS-treated wells using a detection strategy that used a goat anti-rabbit - HRPO complex to detect the polyclonal rabbit anti-CT antibody. Using this detection strategy, the colorimetric change seen in osteoclasts treated with the conjugate appeared to be very very low, if any, and was the same as that seen in osteoclasts treated with PBS (Fig.4.9). The remaining 3 conjugate-treated osteoclast wells and 2 PBS-treated wells were subjected to an ELISA using a second detection strategy that utilized streptavidin-HRPO to detect biotinylated human calcitonin. The colorimetric change seen in these three osteoclast-seeded wells was clearly noticeable and visibly higher than the colorimetric change in osteoclasts treated with PBS.

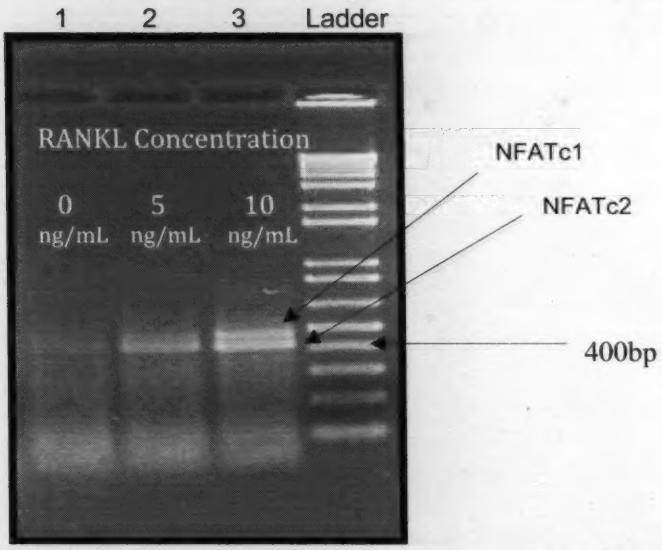


Fig. 4.1: NFATc1 amplification of cDNA showing bands of varying intensity at 400kb following 24hr treatment of RAW 264.7 cells with 20ng/mL of M-CSF and increasing concentrations of RANKL: 0ng/mL, 5ng/mL, 10ng/mL in lanes 1,2 and 3 respectively. Note that the higher of the two 400bp bands in lane 1 is the lightest, the band in lane 2 is more intense, and the band in lane 3 is the most intense. The lower band is that of an isomer, NFATc2.

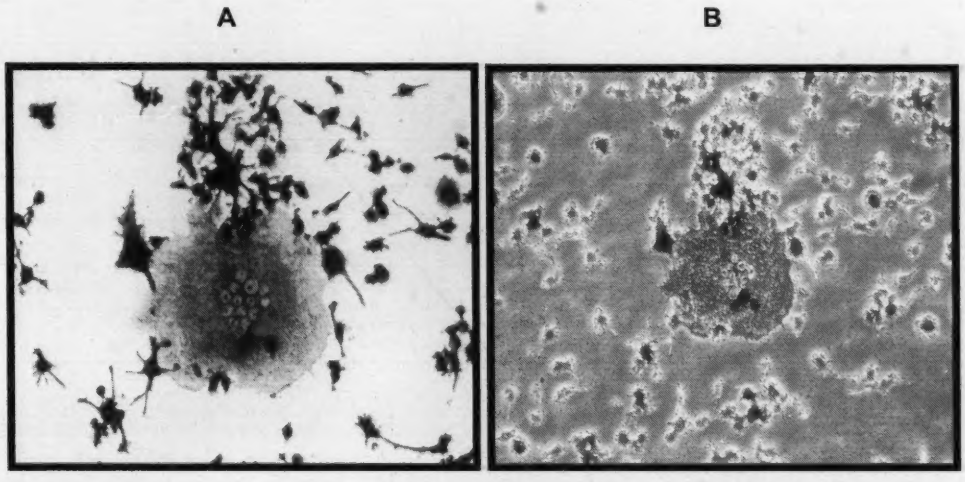


Fig. 4.2: Images of a mature osteoclast that was generated following 7 days of treatment with 20ng/mL of M-CSF and 20ng/mL of RANKL. Clearly visible in both A (regular) and B (phase contrast), is the multi-nucleated nature of the osteoclast as well as the surrounding cytoplasmic membrane. The small darker cells around the osteoclast in both images are partially differentiated or undifferentiated RAW 264.7 cells.

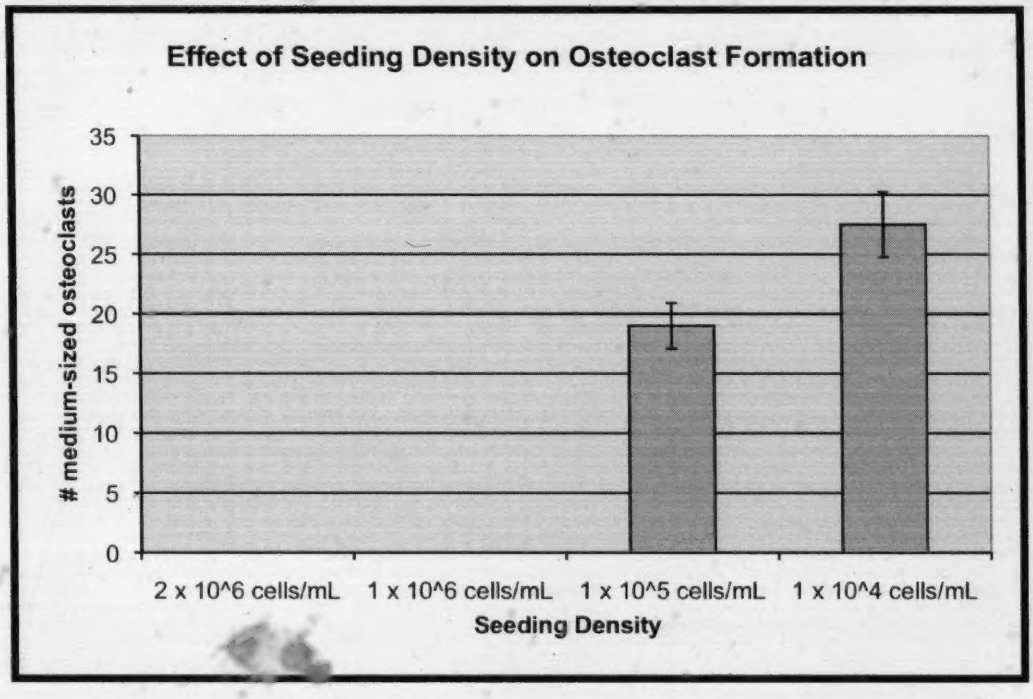


Fig. 4.3: Comparison of the number and size of osteoclasts generated from RAW 264.7 cells seeded at different densities in duplicate and treated with M-CSF and RANKL over the course of 7 days. The average number of medium-sized (<5 nuclei) osteoclasts was a lot higher in cells seeded at 1 x 10^5 cells/mL than in cells seeded at 2 x 10^6 cells/mL and 1 x 10^6 cells/mL. RAW 264.7 cells that were seeded at 1 x 10^4 cells/mL produced the highest number of medium-large osteoclasts (more than double that of 1 x 10^5 cells/mL), and were the only cells to produce very large osteoclasts medium-large osteoclasts (more than double that of 1 x 10^5 cells/mL).

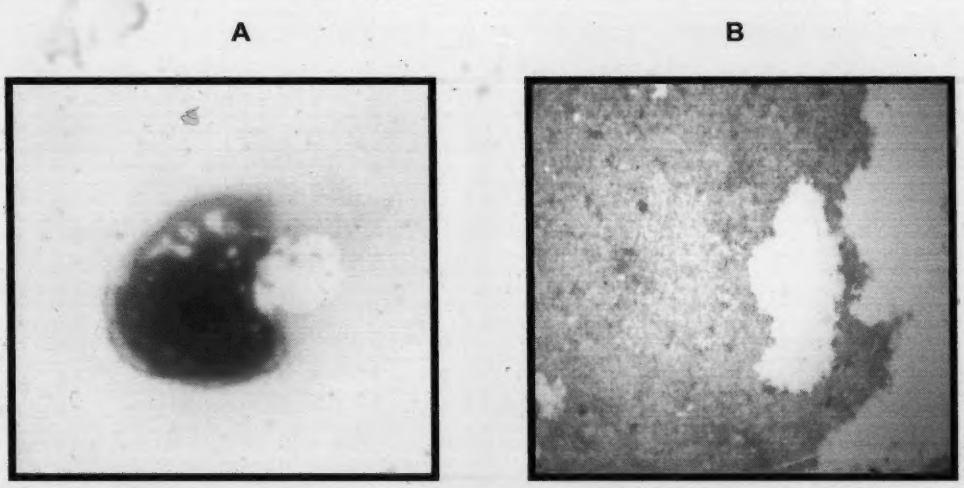


Fig. 4.4: (A) Image of a TRAP-stained osteoclast that is in the process of resorbing calcium phosphate. (B) Toluidine staining of calcium phosphate following the removal of osteoclasts shows clearly the resorption pits (white) formed by osteoclasts in the calcium phosphate (brown) system.

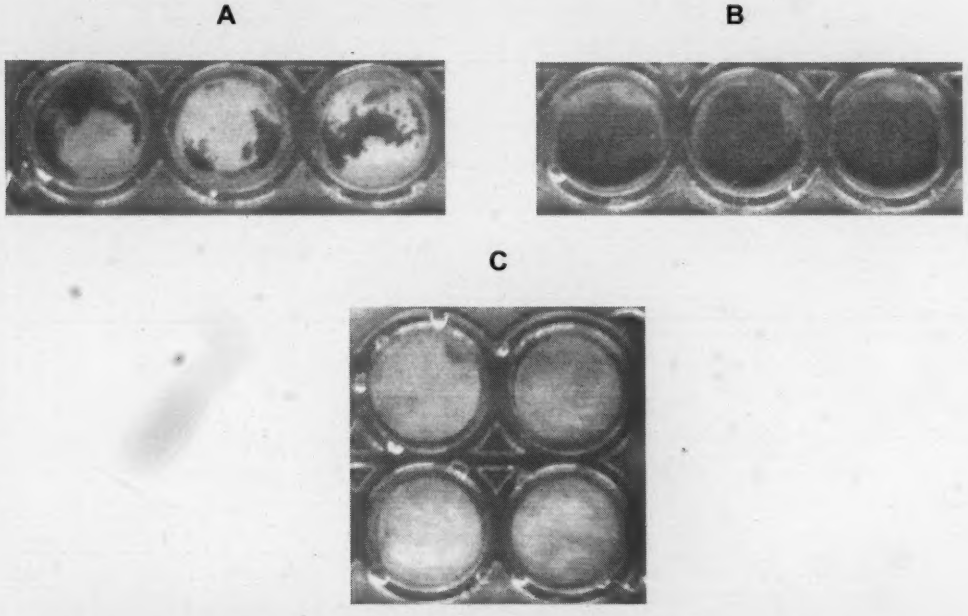
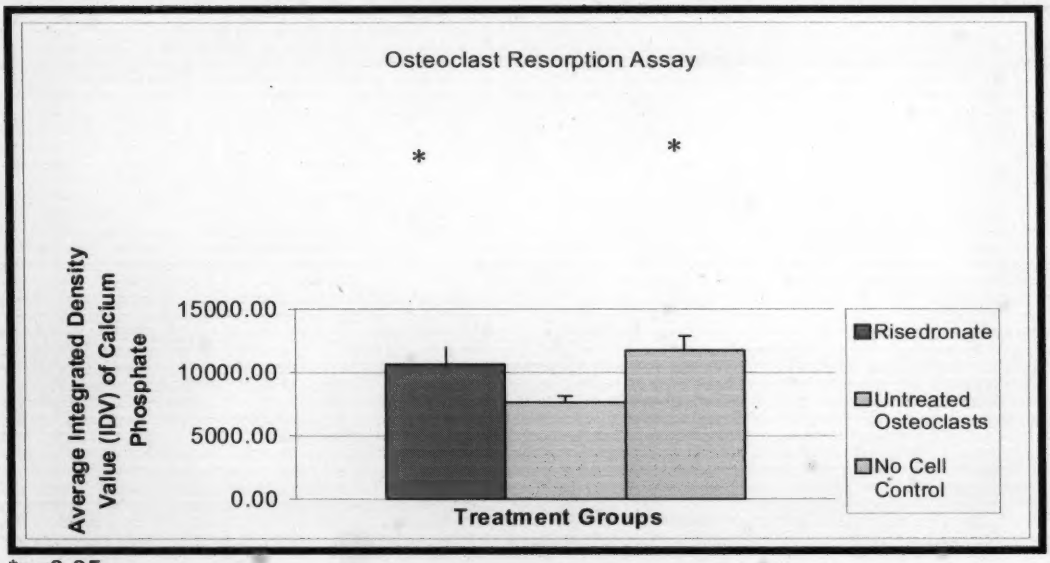


Fig. 4.5: Images showing the calcium phosphate wells following 0.1% toluidine treatment, with the white area being the Calcium Phosphate mineral and the dark areas being the resorptive clearings. (A) Risedronate-treated osteoclast-seeded well: the conservation of calcium phosphate in the three wells appears to be very high. (B) Untreated Osteoclast-seeded wells: There appears to be little to no calcium phosphate remaining these wells where osteoclasts were not treated with the anti-resorptive drug. (C) Unseeded wells (NCON): there appears to be complete conservation of calcium phosphate in these NCON wells.



*p<0.05

Fig. 4.6: Statistical analysis carried out using densitometry measurements showing that osteoclasts treated with Risedronate (n=3) resorbed significantly less calcium phosphate than untreated Osteoclasts (n=3), and that the difference between the conservation of calcium phosphate between Risedronate-treated cells and the no-cell NCON wells (n=3) was not significant.

Sample	Wells	Avg. A650nm	Variance
RAW cells + BSA-PBS (NCON1)	A 1 – A 4	0.044	4.8×10^{-5}
RAW cells + 1 µg Anti-RANK	C 1 – C 4	0.054 *	6.0×10^{-6}
RAW cells + 5 µg Anti-RANK	E 1 – E 4	0.101 *	2.6×10^{-5}
RAW cells + 10 µg Anti- RANK	F 1 – F 4	0.169 *	6.8×10^{-5}

*p<0.05

Table 4.1: Quantitative results of the ELISA performed on RAW 264.7 cells, using spectrophotometry (650nm) to analyze the binding of anti-RANK to RANK on these cells. RAW 264.7 cells treated with 1µg of Anti-RANK showed a small but significant colorimetric change when compared to RAW cells treated with BSA-PBS (NCON1). Cells treated with 5µg of Anti-RANK showed a significant colorimetric change than that seen in NCON1, and those treated with 10µg of Anti-RANK showed a significant change that was nearly quadruple that seen in the NCON1 treatment.

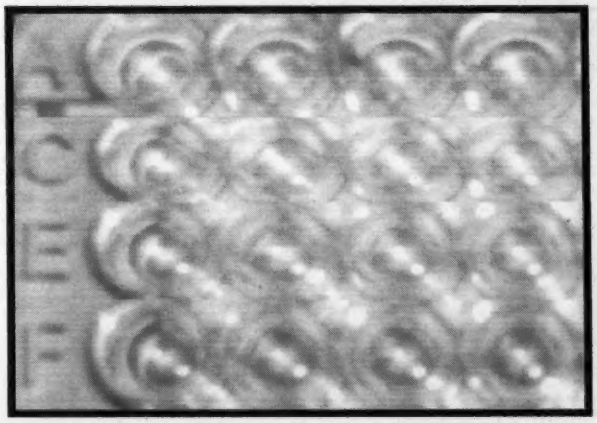


Fig. 4.7: Qualitative results of the ELISA carried out to observe the dose-dependent binding of anti-RANK to RAW 264.7 cells. The cells treated with BSA-PBS (Row A) and those treated with 1mg of Anti-RANK (Row C) show no visible colorimetric change. Cells treated with 5mg of Anti-RANK (Row E) are shown to display a light blue colour, and those treated with 10mg of Anti-RANK (Row F) show a darker and more intense blue colour.

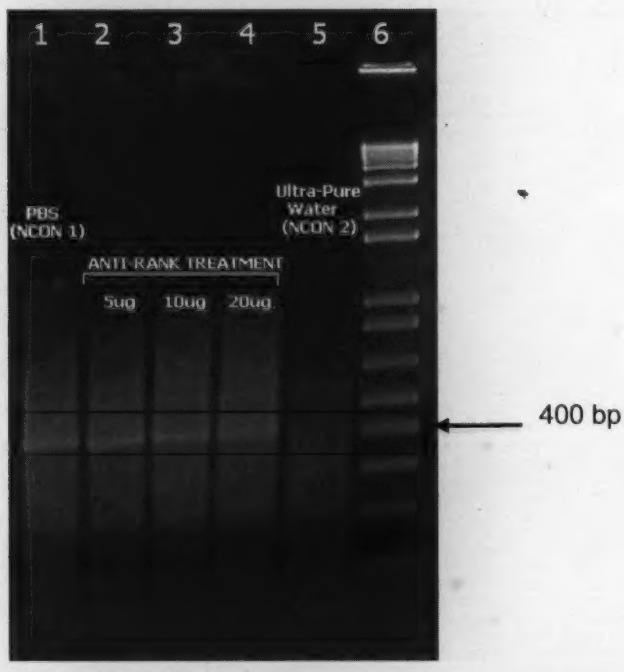


Fig. 4.8: Amplification of NFATc1 from cells treated with BSA-PBS (lane 1), ultra-pure water (lane 5), and varying quantities of anti-RANK. The intensity of the band at ~400bp in cells treated with 5μg (lane 2), 10μg (lane 3) and 20μg (lane 4) appears to be the same as that seen in the two negative controls.

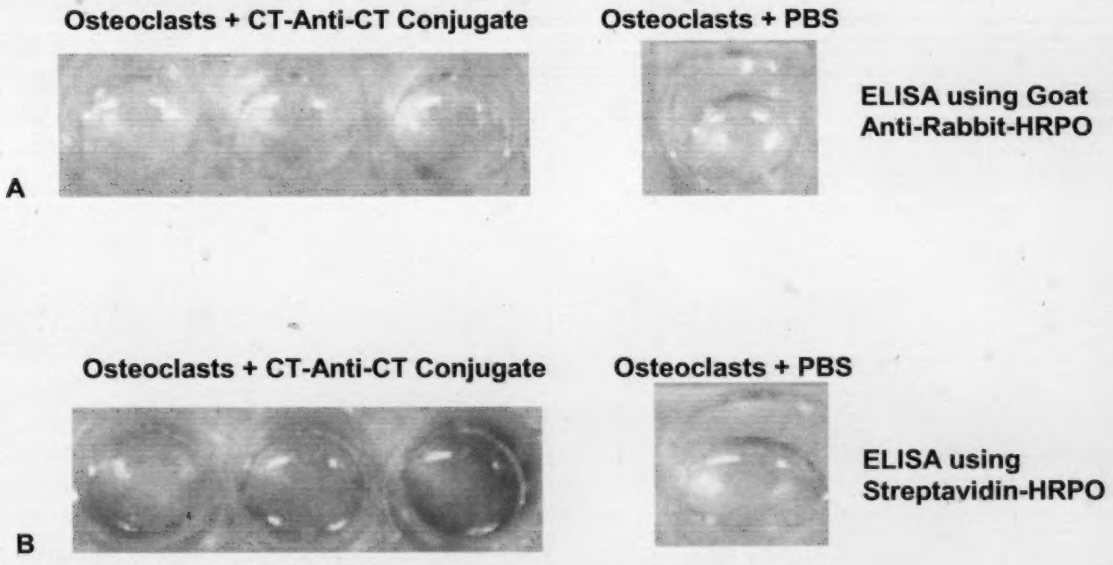


Fig. 4.9: Results of the ELISA conducted on mature osteoclasts treated with the PBS (NCON) and the CT-(Anti-CT) conjugate, as determined qualitatively. (A) The first detection strategy using goat anti-rabbit – HRPO to detect anti-CT in CT-anti-CT treated osteoclast suggests that there is very little, if any, colorimetric when compared to that of the PBS-treated osteoclast control. (B) The second ELISA strategy using streptavidin-HRPO to detect free CT suggests that the colorimetric change is very high in CT-anti-CT treated osteoclasts when compared to the PBS-treated osteoclast control.

4.6 Delivery of the CT-ST-anti-RANK conjugate to osteoclast receptors

Following the generation of the CT-ST-anti-RANK conjugate by the two-step conjugation method, the conjugate was diluted into 1/100 (C1) as well as 1/500 (C2) in order to carry out an ELISA on RANK-coated 96-well plates. To perform the ELISA, 12 wells of a 96-well high-protein binding plate had been coated overnight with BSA (as a control) and another 12 wells were coated with soluble RANK. Then, each dilution of the conjugate was administered in triplicate to the BSA-coated control wells as well as the RANK-coated treatment wells. Free biotinylated-CT and BSA-PBS was added in triplicate to 3 treatment wells and 3 control wells each. Upon detection using a rabbit anti-CT antibody followed by goat anti-rabbit-HRPO, an image was recorded using a digital camera in order to allow for visual analysis (Fig.4.10). In addition, the colorimetric change was measured using the BioTek EL 808 automated plate reader at 650nm (Fig 4.11). There was no colorimetric change seen in BSA-coated wells treated with C1 or C2. This was also the case for BSA-coated wells treated with BSA-PBS, and only a very slight colour change was seen in BSA-coated wells treated with free b-CT. There was also very little colour change in RANK-coated wells that were treated with b-CT, and no colour change in those treated with BSA-PBS. There was however, a significant colorimetic change in RANK-coated wells treated with the C2 dilution of the conjugate when compared to the RANK-coated wells treated with CT. There was also a significant colorimetric change in RANK-coated wells treated with the C1 dilution of the conjugate when compared to RANK-wells treated with free CT.

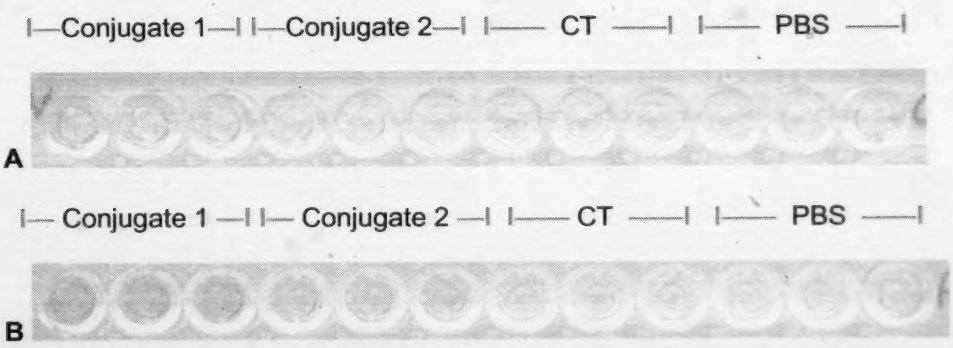
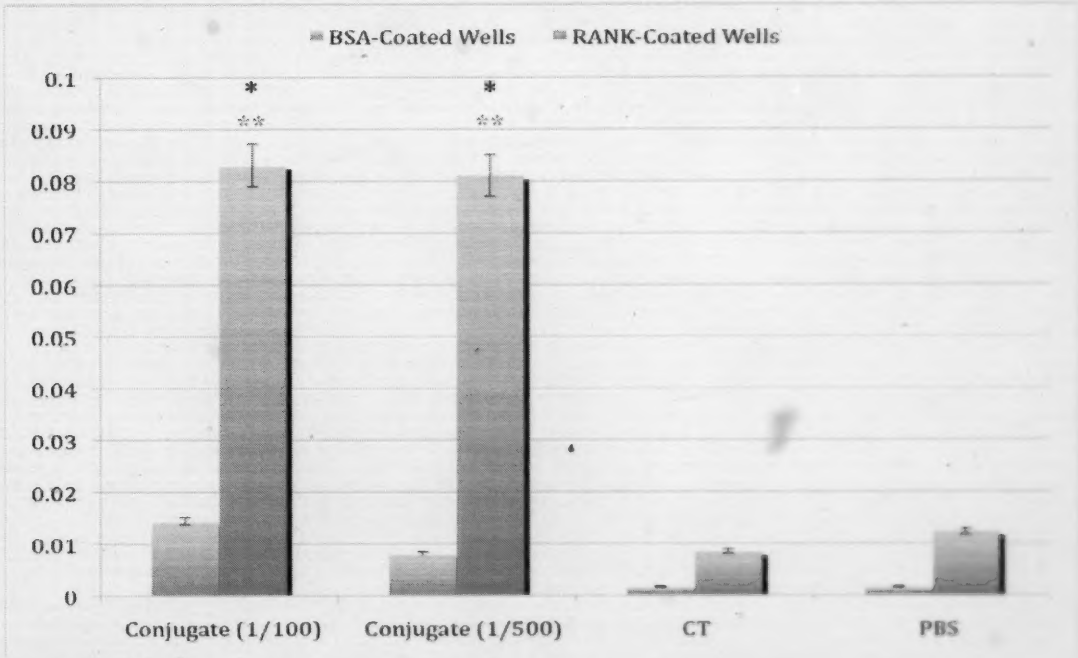


Fig.4.10: Visual confirmation of the increased colorimetric change in RANK-coated wells treated with Conjugate 1(1/100) and Conjugate 2 (1/500) when compared to the BSA-coated wells that received the same treatment. There is also a visibly higher colorimetric change between the conjugate-treated RANK-coated wells when compared to the CT-treated RANK-coated wells.



*p<0.05

Fig. 4.11: Data showing a significant difference between the colorimetric change seen at 650nm between conjugate (1/100 and 1/500)-treated RANK-coated wells when compared to the conjugate (1/100 and 1/500) -treated BSA-coated wells (*). RANK-coated wells treated with conjugate (1/100 and 1/500) also showed significantly higher colorimetric change when compared to RANK-coated wells treated with CT (**)

CHAPTER 5

DISCUSSION

5.1 CONCLUSION

Yoshida *et al.* (1990) and Kong *et al.* (1999) were the first researchers to find that the presence of M-CSF and RANKL, respectively, was crucial to the formation of mature osteoclasts in mice. In the case of RAW 264.7 cells, which are murine in origin, Hsu *et al.* (1994) confirmed that RANKL was essential for the promotion of osteoclastogenesis. With regards to M-CSF however, studies have suggested that while RAW 264.7 cells do express the c-fms receptor (Shadduck *et al.*, 1993), they may also express M-CSF (Osdoby *et al.*, 2003) and therefore M-CSF supplementation may not be critical for the triggering of osteoclastogenesis in RAW 264.7 cells. M-CSF is a permissive factor however, and the addition of more M-CSF results in a higher expression of RANK (Hsu *et al.*, 1999). Based on this, we chose to treat RAW 264.7 cells with 25ng/mL of M-CSF in addition to RANKL in an effort to further increase RANK expression and thereby further promote osteoclastogenesis. Upon treatment of RAW 264.7 cells with RANKL and M-CSF, we found that osteoclastogenesis was successfully triggered in RAW 264.7 cells as evidenced by the up-regulation of the downstream protein NFATc1 when compared to untreated RAW 264.7 cells which showed a basal level of NFATc1 expression. NFATc1, according to Day *et al.* (2004), is one of the main nuclear factors to be regulated by RANKL during osteoclastogenesis. NFATc1 has also been shown to be essential for the induction of osteoclast genes (Ishida *et al.*, 2002; Takayanagi *et al.*, 2002). Therefore, the upregulation of NFATc1 is a good indicator of successful osteoclastogenesis. Additionally, there was a clear dose-dependent upregulation of NFATc1 seen in cells that were treated in quadruplicate with 0ng/mL, 5ng/mL and 10ng/mL of RANKL. We are confident that the dose-dependent increase of NFATc1 levels seen following treatment with RANKL confirms that

osteoclastogenesis has indeed been triggered 24hrs after dosing (Fig.4.1). To confirm that this triggering of osteoclastogenesis would lead to the formation of mature osteoclasts, dosing with RANKL and M-CSF was carried out over the course of a week. This resulted in the formation of a number of osteoclasts that were visualized by staining for the enzyme tartrate-resistant acid phosphatase (TRAP). TRAP has been used as a histochemical marker of osteoclasts for several decades (Burstone, 1959), and is found in cells of the monocytic macrophage lineage such osteoclasts and dendritic cells (Hayman *et al.*, 2000). The staining for TRAP activity is a technique commonly used to visualize osteoclasts, and was used an indicator of osteoclastogenesis by Nicolin et al. (2005). The principle behind staining for TRAP activity involves the use of napthol AS-BI phosphates in conjunction with diazonium salts for the detection of acid phosphatase, and it was first used in leukocytes by Goldberg and Barka (1962). According to Sigma-Aldrich, the Acid Phosphatase Leukocyte kit used in this study introduces tartrate along with a napthol AS-BI phosphate, which upon contact with TRAP is enzymatically hydrolyzed resulting in the release of napthol AS-BI (7-Bromo-3-hydroxy-2-naphtho-o-anisidine). Napthol AS-BI then couples with the stable diazonium fast garnet GBC salt that is formed in the presence of sodium nitrite in an acidic medium, resulting in the formation of insoluble maroon deposits at sites of acid phosphatase activity. Cells containing tartrate-sensitive acid phosphatase are devoid of enzymatic activity due to the introduction of tartrate into the system in the first step, and therefore do no show any maroon deposits in the cells. Following the staining for TRAP activity, hematoxylin is added to the system in order to stain all TRAP-negative cells a dark blue/black colour. This enables easy visual identification of osteoclasts against a background of undifferentiated RAW 264.7 cells (Fig. 4.2).

Following the successful growth, propogation and visualization of osteoclasts, we wanted to determine the ideal seeding density of RAW 264.7 cells for osteoclast generation, using the existing literature as a guideline. RAW 264.7 cells were first seeded onto a 24-well plate in quadruplicate at 2 x 10^6, 1 x 10^6, 1 x 10^5, and 1 x 10^4 cells/mL. They were then treated with M-CSF and RANKL over the course of a week and stained for TRAP activity. The resulting TRAP-positive cells were qualitatively classified as being either medium-sized (<5 nuclei) osteoclasts or very large (>4 nuclei) osteoclasts depending on the number of nuclei in each multinucleated cell and the expanse of their cytoplasm. The number of medium-sized and very large osteoclasts in each well was counted under a microscope, and the average number of medium and very large osteoclasts in each set of treatment wells was recorded (Fig.4.3). The results show that the largest numbers of medium-sized osteoclasts were seen at the lowest of the four seeding densities (1 x 10^4 cells/mL). This was also the only seeding density that showed any very large osteoclasts. Our results suggest that after the 1 x 10^4 cells/mL seeding density, the higher the seeding density of RAW 264.7 cells, the lower the number and lesser the size of osteoclasts generated *in vitro* (Fig.4.3). We believe that this is as a result of there not being enough nutrients for cell sustenance at higher seeding densities, as Mauck *et al.* (2003) found to be the case with chondrocyte growth. Lower seeding density also minimizes contact inhibition As described by Nelson *et al.* (2004), contact inhibition is a phenomenon wherein cell-to-cell contact in a culture prevents cell spreading and decreases cell adhesion to an extracellular matrix. Therefore, it seems likely that seeding RAW 264.7 cells at a high density results in a large number of cells becoming detached very soon after seeding as a result of contact inhibition. The seeding density that we found to be ideal is also comparable to

the findings of Yang *et al.* (2006), who suggested plating RAW 264.7 cells at 0.5-0.6 x 10^4 cells/mL. In all subsequent experiments requiring osteoclast formation from RAW 264.7 cells, the seeding density was 1 x 10^4 cells/mL. The next step in our study was to determine whether the osteoclasts generated *in vitro* were functional and capable of bone resorption, and whether treatment with a bisphosphonate would result in a halting of resorption.

In order to test the efficacy of the osteoclasts as well as the effect of bisphosphonates on their function, an experiment was designed so as answer both questions in one assay. Osteoclasts were first grown in a 25cm^2 flask, counted, and then seeded onto 6 wells of commercially available slides that were coated with osteologic material (calcium phosphate). Three other wells were left empty to serve as a negative control against degradation of calcium phosphate by the media. For the first three days, all 9 wells were dosed every 48hrs with M-CSF (25ng/mL) and RANKL (25ng/mL). After 3 days, one set (3 wells) of osteoclast-coated wells was dosed with Risedronate (50μM) while the other set of osteoclast-coated wells was left untreated. All 9 wells were still dosed with M-CSF and RANKL (and Risedronate in the treatment set) every 48hrs. On day 8, we used a digital camera connected to a microscope to record images of osteoclasts in the process of resorbing the calcium phosphate (Fig. 4.4 A). Then, osteoclasts were washed off using bleach, and the osteologic material was stained using toluidine blue as described by (Kawai *et al.*, 2006) in order to increase the contrast between resorption clearings and un-resorbed osteologic material. Images of all wells were recorded using a camera connected to a microscope (4.4 B). We found that the osteoclasts successfully resorbed the calcium phosphate coat on the wells, and using the Image J software, we were able to statistically analyze the percentage of calcium phosphate conserved in

each of the 9 wells. The results showed that the amount of osteologic material resorbed by the osteoclast-seeded wells that did not receive Risedronate treament was significantly higher than that seen in wells containing osteoclasts that were treated with Risedronate. In fact, Risedronate-dosing was so successful that the amount of calcium phosphate resorbed was not significantly different than that seen in control wells that were not even seeded with cells. These results suggest that the osteoclasts grown in this study were indeed functional and susceptible to bisphosphonates. As a result, we have now developled an *in vitro* resorption assay that can be used to conduct efficacy studies for current as well as future novel anti-resorptive therapies.

Following the establishment of RAW 264.7 and osteoclast cell culture techniques, we wanted to test the validity of our strategy to deliver calcitonin into an osteoclast microenvironment using a congjuate targeting the receptor RANK on osteoclasts.

Our first sub-hypothesis was that the surface receptor RANK on osteoclast precursor RAW 264.7 cells could be targeted by anti-RANK in a dose-dependent fashion, and that this binding would not trigger any osteoclastogenesis. To test this, we first seeded RAW 264.7 cells into 16 wells overnight and divided the wells into fours sets of four wells. The first set of wells was treated with BSA-PBS, and served as a negative control (NCON1) while the other three sets of wells were treated with 1mg, 5mg and 10mg of anti-RANK respectively. The results show that the binding of anti-RANK to its ligand (RANK) was successful in all three anti-RANK treatments, and the colorimetric change seens in the ELISAs showed that the binding of anti-RANK to RANK on RAW 264.7 cells in all three treatment sets was significantly higher than the negative

control. We also showed that there was significant dose-dependency in the binding of anti-RANK to RAW 264.7 cells (Table 4.1). We then wanted to ensure that the binding of anti-RANK to RAW 264.7 cells did not result in any triggering of osteoclastogenesis. To achieve this, we seeded RAW 264.7 cells into 16 wells of a 24-well plate, and dosed them in sets of 4 with BSA-PBS (NCON1), 1μg, 5μg, and 10μg of anti-RANK. Following a 24hr incubation period with the varying amounts of anti-RANK, the RNA from the 4 wells of each treatment set were pooled, converted to cDNA, and had the NFATc1 gene amplified using PCR. The result showed that there was the same, low basal expression of NFATc1 in all four wells, suggesting that there was no upregulation of the osteoclastogenic marker NFATc1 following anti-RANK treatment of RAW 264.7 cells (Fig. 4.8). This can be compared and contrasted to Fig. 4.1, which shows NFATc1 upregulation. Even though these studies were carried out on RAW 264.7 cells and not osteoclasts, the results are very important since RANK is found on mature osteoclasts as well as their precursors (Anderson *et al.*, 1997). Therefore, based on the successful binding of anti-RANK to RANK on osteoclast precursor (RAW 264.7) cells, we believe that the binding of anti-RANK to RANK on mature osteoclasts will also be successful and non-osteoclastogenic. We believe that anti-RANK can therefore be used as a targeting tool against osteoclasts, and this would form the 'targeting' arm of our final anti-resorptive CT-ST-anti-RANK conjugate.

Our second sub-hypothesis was that the treatment of osteoclasts with calcitonin (CT) molecules conjugated to anti-CT antibodies would result in the delivery of CT to the high affinity calcitonin receptors. We also wanted to investigate whether the carrier anti-CT antibody remains attached to its CT 'pay-

load' following CT's interaction with its high affinity receptor (CTR) on osteoclasts. To do this, we grew osteoclasts in 8 wells of a 24-well plate and treated 6 of these wells with the CT-(anti-CT) conjugate, while the other 2 wells were treated with just PBS. An assay was designed to look for CT and anti-CT using two different detection strategies. The logic behind this dual-detection design was that detecting for CT as well as anti-CT would let us know two things: firsty, it would show us whether the CT able to bind to its receptor and stay attached; secondly, it would let us known whether the CT-(anti-CT) conjugate becomes dissociated into CT and anti-CT following introduction into the system. In order to conduct 2 different ELISAs, we split the 6 wells treated with CT-(anti-CT) into two groups of 3, and also split up the two PBS-treated osteclast wells. One set of wells (3 treatment wells + 1 NCON1) was treated with a streptavidin-HRPO molecules that detected the biotin tag on free CT, while the other set of wells was treated with a goat-(anti-rabbit)-HRPO molecule that would detect any rabbit anti-CT molecules present in the system. The results (Fig. 4.5) suggest that while CT is present in the system, there is little sign of any anti-CT. This is very intriguing, as it suggests that following the interaction between CT and its receptor (CTR) on osteoclasts in the system, there was a dissociation of the CT-(anti-CT) conjugate. In other words, it appears as though the affinity of the CTR for its ligand CT is greater than the affinity of anti-CT for CT. To understand this better, one needs to look at the equilibrium constants of CT-CTR and CT-(anti-CT) binding. According to the findings of Shyu *et al.* (1996), the dissociation constant (Kd) of hCT-CTR is ~3nM while the Kd of a typical antibody-ligand conjugate ranges from 1μm (high chance of dissociation) to 1nM (low chance of dissociation). In other words, the dissociation constant of CTR-CT is as low as some of the lowest antibody-antigen dissociation constants known. Also, the

inverse of Kd is known as the association constant (Ka), and describes the likelihood of two molecules associating with one another. Since Kd = 1/Ka, any complex having a low Kd also has a high Ka. So, when CT is brought in close proximity to its receptor by a polyclonal carrier antibody with a Kd lower than ~3nM, it is very likely to dissociate from the antibody and associate with its receptor which has a higher Ka. Based on the results that show that CT did indeed bind more readily to CTR rather than anti-CT, it is likely that the CT-(anti-CT) interaction has a Kd higher than 3nM. It is also of note that Shyu *et al.* (1996) found that salmon CT has a 10-fold lower dissociation constant than human CT, or in other words, has a 10-fold higher affinity for CTR than hCT. Overall, the results of the CT-(anti-CT) study confirm that anti-CT molecules could potentially be used as the 'carrier' arm of an anti-resorptive conjugate to successfully deliver the CT 'pay-load' to high-affinity CT receptors on the surface of cells targeted by the anti-RANK 'targeting' arm.

Our final sub-hypothesis was that the construction of a conjugate carrying biotinylated calcitonin, while being attached to an anti-RANK via streptavidin, would result in the targeting of CT to the microenviroment of cells displaying RANK. To construct this conjugate, we exploited the extraordinary binding affinity between streptavidin and biotin (10^{-15}M) to bring the targeting and carrier arms together. In this study, however, we decided to directly conjugate biontinylated CT to streptavidin instead of using anti-CT as a carrier. The reason for this is that this study was carried not carried out on osteoclasts or RAW 264.7 cells, but instead on a surface that mimicked the expression of RANK on these cells. Since there are no calcitonin receptors on this surface, using anti-CT to carry CT for delivery does not justify the cost and complexity associated with the presence of a second antibody in the conjugate. The coating of a high protein-affinity 96-well

plate with soluble RANK allowed for the mimicking of the presence of RANK on osteoclasts. Like calcitonin, the anti-RANK antibody was also purchased in a biotinylated form, as this would allow for its conjugation to streptavidin. The use of streptavidin for the conjugation of an antibody is not uncommon, and was carried out successfully by Shuvaev *et al.* (2004). They used streptavidin to conjugate a catalase therapeutic to an antibody that recognized a certain receptor on endothelial cells. Their streptavidin conjugate, much like ours, was intended purely as a proof-of-principle.

In addition to coating 12 wells with RANK, we also coated 12 wells with an equal amount of BSA (1μg/well). This serves as a control against non-specific binding of the conjugate to the high protein-binding 96-well plate. The 12 treatment and 12 control wells were divided into sets of 3 wells so as to allow for treatments in triplicate. Following the blocking of non-specific binding sites in all 24 wells with 2% BSA, the first two sets of wells in both the treatment and the control rows were treated with 1/100 and 1/500 dilutions of the conjugate. Also, since we did not purify the conjugate to get rid of excess CT from the reaction mixture, we installed a CT control. This involved the treatment of one set (3 wells) of RANK-coated and one set of BSA-coated wells with an excess of free biotinylated-CT. The last set of wells in the RANK-coated and BSA-coated wells were treated with only BSA-PBS. All four treatments were carried out for the same amount of time before we detected for CT in the system using an ELISA. The detection mechanism used was a streptavidin-HRPO molecule that would detect for any CT in the system. We found that colorimetric change seen in the RANK-coated wells treated with the conjugate was significantly higher than that seen in the BSA-coated wells treated with the conjugate (Fig.4.10; Fig.4.11). Upon measuring the colorimetric change, the conjugate treated RANK-coated

wells had a significantly higher signal when compared to the RANK-coated wells that were treated with free b-CT (Fig.4.11). The visual analysis of this assay shows that there was a very slight signal from RANK-coated wells that were treated with CT, and this can be attributed to the fact that the 96-well plate used was specifically picked for its very high affinity for proteins, and CT being a very small molecule, probably managed to bind in small amounts. However, as seen in Fig. 4.10, this signal was very weak. We did not witness this weak signal from conjugate-treated BSA-coated wells because the conjugate is very large, and most likely too large to effectively attach itself to the surface of wells that were already coated in such a short perdiod of time. Therefore we can safely state that the positive CT signal was as a result of conjugate-bound CT and not free unbound CT.

This assay showed that CT could indeed be brought into the vicinity of RANK using a CT-ST-anti-RANK conjugate. This, along with all of the previously discussed results involving the delivery of CT by anti-CT molecules, confirms our general hypothesis that the osteoclast surface receptor RANK can be used as a target to deliver therapeutic anti-resorptive peptides such as calcitonin directly to osteoclasts.

5.2 Future Directions

In this study we showed that the delivery of a therapeutic drug (CT) directly onto osteoclasts is a strategy that is feasible and so we recommend that future studies be conducted by modifying the calcitonin-delivering proof-of-principle conjugate in a way that increases its targeting and antiresorptive capabilities, as well as its efficacy *in vivo*. While we did not construct a conjugate that had an anti-CT arm loaded with CT, based on earlier results, we can safely

predict that the replacement of the biotinylated-CT with a biotinylated anti-CT molecule carrying CT would result in the successful delivery of CT to its receptors on osteoclasts. So the immediate future should involve the formation of a conjugate that has CT-(anti-CT) as one arm and anti-RANK as the other arm, with both linked via streptavidin. The conjugate should then be tested on osteoclasts *in vitro* to confirm that the conjugate targets RANK on osteoclasts and then successfully delivers the CT onto high-affinity CTRs on these cells. We also propose that a resorption assay be conducted as described earlier in theis study to prove that the targeted delivery of CT using the conjugate does in fact result in the halting of resorption. Once the anti-resorptive efficacy of the conjugate has been established, we recommend that the molecule be modified to allow for testing *in vivo*.

The presence of two large antibody arms on the CT-(anti-CT)-ST-(anti-RANK) conjugate makes it very bulky and impractical for *in vivo* applications due to steric and immunogenic implications. So we propose that much smaller molecules that retain the same antigen recognition should replace these bulky antibodies. The ideal candidates for this are the very small recombinant single-chain variable fragment (scFv) molecules. These molecules are constructed to consist of only the small antigen-recognition region of a full-sized antibody, and have the same antigen specificity as a whole monoclonal antibody. The main advantage of recombinant scFvs is that since lack the large immunogenic Fc portion that full-sized antibodies contain, they are a lot better suited to in vivo applications. For instance, Cheng and Allen (2008) generated monoclonal antibodies and scFv particles that recognized the receptor CD19 on B-cells and attached each of them onto stealth liposomes loaded with doxorubicin in order to facilitate targeted delivery of the drug to malignant B-cells. They found that both

the monoclonal-coated and scFv-coated liposomes delivered the drug to the target cells, but the authors preferred the scFv-coated liposomes due to the decreased presence of foreign peptdes on scFv particles as a result of there being no Fc domain, and therefore the low immunogenicity of the compound. Therefore, if we were to substitute the bulky monoclonal anti-RANK and polyclonal anti-CT with anti-RANK and anti-CT scFv molecules, this conjugate would be instantly made lean and more effective *in vivo*.

Secondly, the large streptavidin connector molecule will also need to be modified in order to allow for *in vivo* delivery of the conjugate. Studies have found that the *in vivo* distribution of streptavidin is not uniform, and results in the sequestering of streptavidin in the kidneys (Schechter *et al.*, 1990; Zhang *et al.*, 1997). Wilbur *et al.* (1998) found that chemical succinylation of streptavidin improved the distribution characteristics of the molecule, thus making it more favourable for *in vivo* targeting applications. We therefore propose the use of succinylated streptavidin in place of the streptavidin used in the construction of our conjugate so as to make it more efficacious *in vivo*.

Lastly, this study used human CT as the drug payload instead of salmon CT. We recommend that the conjugate be modified to carry salmon calcitonin in place of human calcitonin as this will result in greater efficacy as well as easier delivery since sCT has a higher affinity for human CTRs.

One the whole, we believe that calcitonin as a therapeutic holds a lot of promise, and that the targeted delivery of this molecule to osteoclasts has the potential to make CT a safe and effective frontline treatment for postmenopausal osteoporosis.

CHAPTER 6

REFERENCES

1. Abou-Samra, A. B., Juppner, H., Force, T., Freeman, M.W., Kong, X.F., Schipani, E., Urena, P., Richards, J., Bonventre, J.V., Potts, J.T., Kronenberg, H.M., and Segre, G.V. 1992. Expression cloning of a common receptor for parathyroid hormone and parathyroid hormone-related peptide from rat osteoblast-like cells - a single receptor stimulates intracellular accumulation of both cAMP and inositol trisphosphates and increases intracellular free calcium. *Proc Natl Acad Sci USA* **89**: 2732-2736.
2. Agency for Healthcare Research and Quality. 2001. Osteoporosis in Postmenopausal Women: Diagnosis and Monitoring. Summary, Evidence Report/Technology Assessment: Number 28. AHRQ Publication Number 01-E031, February 2001. AHRQ, Rockville, MD. http://www.ahrq.gov/clinic/epcsums/osteosum.htm. Accessed on April 23rd, 2009.
3. Agus, Z. S., Wasserstein, S., Goldfarb, S. 1981. PTH, calcitonin, cyclic nucleotides and the kidney. Ann Rev Physiol **43**: 583.
4. Akisaka, T., Gay, C. V. 1986. Ultracytochemical evidence for a proton pump adenosine trisphosphate in chick osteoclasts. Cell Tissue Res **245**: 507–512.
5. Albagha, O.M., et al. 2005. Association of oestrogen receptor alpha gene polymorphisms with postmenopausal bone loss, bone mass, and quantitative ultrasound properties of bone. J. Med. Genet. **42**: 240–246.
6. Alessi, D.R., Kozlowski, M.T., Weng, Q.P., Morrice, N., and Avruch, J. 1998. 3- Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. Curr. Biol. **8**: 69–81.
7. Alevizaki, M., Shiraishi, A., Rasool, F. V., Ferrier, J. G. M., MacIntyre, I., and Legon, S. 1986.The calcitonin-like sequence of the beta-CGRP gene. Fed Eur Bone Soc Lett **206**:47–52.

8. Andersen, T.L., del Carmen Ovejero, M., Kirkegaard, T., Lenhard, T., Foged, N.T., Delaissé, J.M. 2004. Bone **35**: 1107–1119.

9. Anderson, D.M., et al. 1997. A homolog of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature.* **390**: 175–179.

10. Angel, N.Z., Walsh, N., Forwood, M.R., Ostrowski, M.C., Cassady, A.I., Hume, D.A. 2000. J. Bone Miner. Res. **15**: 103–110.

11. Antonin, K.H., Saano, V., Bieck, P., Hastewell, J., Fox, R., Lowe, P., Mackay, M. 1992. Colonic absorption of human calcitonin in man. Clin. Sci. **83**: 627–631.

12. Arai, F., et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. 1999. J Exp Med **190**:1741–1754.

13. Aronica, S.M., Katzenellenbogen, B.S. 1993. Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol Endocrinol 7:743–752.

14. Asagiri, M., Sato, K., Usami, T., Ochi, S., Nishina, H., Yoshida, H., Morita, I., Wagner, E.F., Mak, T.W., Serfling, E., Takayanagi, H. 2005. Autoamplification of NFATc1 expression determines its essential role in bone homeostasis. J Exp Med **202** (9): 1261-1269.

15. Banchereau, J., Steinman, R.M. 1998. Dendriticcells and the control of immunity. Nature **392**: 245–252.

16. Baron, R., Chakraborty, M., Chatterjee, D., Horne, W., Lomri, A., Ravesloot, J.H. 1993. Biology of the osteoclast. In: Mundy GR, Martin TJ, editors. Physiology and pharmacology of bone. Berlin: Springer- Verlag. p.111–147.

17. Barrett-Connor, E., Mosca, L., Collins, P., Geiger, M.J., Grady, D., Kornitzer,

M., et al. 2006. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. N Engl J Med **355**: 125–37.

18. Baylies, M.K., Bate, M., Ruiz-Gomez, M. 1998. Myogenesis: a view from Drosophila. Cell. **93**: 921–7.
19. Beaudreuil, J., et al. 1995. Short-term local injections of transforming growth factor- β1 decrease ovariectomy-stimulated osteoclastic resorption in vivo in rats. J. Bone Miner. Res. **10**: 971-977.
20. Becker, B.J., and Gay, C.V. 1990. J. Bone Miner. Res. **5**: 569-579.
21. Beglinger, C., Born, W., Muff, R., Drewe, J., Dreyfuss, J.L., Bock, A.; Mackay, M., Fischer, J.A. 1992. Intracolonic bioavailability of human calcitonin in man. Eur. J. Clin. Pharmacol. **43**: 527–531.
22. Bellido, T., Jilka, R.L., Boyce, B.F., *et al.* 1995. Regulation of interleukin-6, osteoclastogenesis, and bone mass by androgens. The role of the AR receptor. J. Clin. Invest. **95:** 2886–2895.
23. Benz, D.J., Haussler, M.R., Thomas, M.A.,*et al.* 1991. High-affinity AR binding and androgenic regulation of alpha 1(I)-procollagen and transforming growth factor-beta steady state messenger ribonucleic acid levels in human osteoblast-like osteosarcoma cells. Endocrinology **128:** 2723–2730.
24. Bernard-Poenaru, O., Roux, C., Blanque, R., *et al.* 2001. Bone-resorbing cytokinesfrom peripheral blood mononuclear cells after hormone replacement therapy: a longitudinal study. Osteoporos. Int. **12:** 769–776.
25. Binkley, N., Bilezikian, J.R., Kendler, D.L., et al. 2006. Official positions of the International Society for Clinical Densitometry and executive summary of the 2005 Position Development Conference. J Clin Densitom **9**: 4-14.
26. Blair, H.C., Teitelbaum, S.L., Ghiselli,R., Gluck, S. 1989. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science **245**: 855-887.

27. Blavier, L., Delaissé, J.M. 1995. Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. J Cell Sci **108**: 3649 –3659.

28. Block, G.A., Raggi, P., Bellasi, A., Kooienga, L., Spiegel, D.M. 2007. Mortality effect of coronary calcification and phosphate binder choice in incident hemodialysis patients. Kidney Int **71**: 438–41.

29. Bolland, M.J., Barber, P.A., Doughty, R.N., Mason, B., Horne, A., Ames, R., et al. 2008. Vascular events in healthy older women receiving calcium supplementation: randomised controlled trial. BMJ **336**: 262–6.

30. Bone, H.G., McClung, M., Verbruggen, N., Rybak-Feiglin, A., DaSilva, C., Santora, A.C., et al. 2007. A randomized double-blind, placebo-controlled study of a cathepsin K inhibitor in the treatment of postmenopausal women with low BMD: one year results. J Bone Mineral Res **22**: s37.

31. Bossard, M.J., Tomaszek, T.A., Thompson, S.K., Amegadzie, B.Y., Hanning, C.R., Jones, C., Kurdyla, J.T., McNulty, D.E., Drake, F.H., Gowen, M., Levy, M.A. 1996. J. Biol. Chem. **271**: 12517–12524.

32. Bourne, H.R., Sanders, D.A., McCormick, F. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349**:117–127.

33. Boyle, W.J., Simonet, W.S., Lacey, D.L. 2003. Osteoclast differentiation and activation. Nature **423**: 337–342

34. Braidman, L.P., Baris, C., Selby, P.L., *et al.* 2000. Preliminary report of impaired oER receptor-alpha expression in bone, but no involvement of AR receptor, in male idiopathic osteoporosis. J. Pathol. **192:** 90–96.

35. Breimer, L.H., MacIntyre, I., Zaidi, M. 1998. Peptides from the calcitonin genes: molecular genetics,structure and function. Biochem.J. **255**: 377-390.

36. Bühling, F., Röcken, C., Brasch, F., Hartig, R., Yasuda, Y., Saftig, P., *et al.* 2004. Pivotal role of cathepsin K in lung fibrosis, *Am J Pathol* **164**: 2203–2216.

37. Burge R, Dawson-Hughes B, Solomon DH, et al. 2007. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005–2025. J Bone Miner Res 2007; **22**(3): 465–75.

38. Burger, E.H., Van der Meer, J.W., van de Gevel, J.S., Gribnau, J.C., Thesingh, G.W., van Furth, R. 1982. J. Exp. Med. **156**: 1604-1614.

39. Burstone, M.S. 1959. Histochemical demonstration of acid phosphatase activity in osteoclasts. J Histochem Cytochem **7**: 39–41.

40. Bush, T.L., Wells, H.B., James, M.K., Barrett-Connor, E., Marcus, R., Greendale, G., et al. 1996. Effects of hormone therapy on bone mineral density—results from the postmenopausal estrogen/progestin interventions (PEPI) trial. JAMA **276**: 1389–96.

41. Campion, J.M., Maricic, M.J. 2003. Osteoporosis in men. Am Fan, Phys. **67**:1521-1526.

42. Caveggion, E., Continolo, S., Pixley, F. J., Stanley, E. R., Bowtell, D. D., Lowell, C. A., and Berton, G. 2003. J. Cell. Physiol. **195:** 276–289.

43. Chabadel, A., Bañon-Rodriguez, I., Cluet, D., Rudkin, B.B., Wehrle-Haller, B., Genot, E., Jurdic, P., Anton, I.M., Saltel, F. 2007. Mol. Biol. Cell **18**:4899–4910.

44. Chabre, O., Conklin, B. R., Lin, H. Y., Lodish, H. F., Wilson, E., Ives, H. E., Catanzariti, L., Hemmings, B. A., Bourne, H. R. 1992. A recombinant calcitonin receptor independently stimulates 3', 5'-cyclic adenosine monophosphate and Ca2+/inositol phosphate signaling pathways. Mol Endocrinol **6**: 551–556.

45. Chambers, T.J. 1982. Osteoblasts release osteoclasts from calcitonin-induced quiescence. Journal of Cell Science **57**: 247-260.

46. Chambers, T. J., Magnus, C. J. 1982. Calcitonin alters behaviour of isolated osteoclasts. J Pathol **136**: 97–106.

47. Chambers, T.J., and Moore, A. 1983. The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. J. Clin. Endocrinol. Metab. **57**: 819-824.

48. Chambers, T. J., Fuller, K., Darby, J. A. 1987. Hormonal regulation of acid phosphatase release by osteoclasts disaggregated from neonatal rat bone. J Cell Physiol **132**: 90–96.

49. Chan, F.K. *et al.* 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science* **288:** 2351–2354.

50. Chan, F.K., Siegel, M.R., Lenardo, M.J. 2000. Signaling by the TNF receptor superfamily and T-cell homeostasis. *Immunity* **13:** 419–422. Chang, S. L., Hoffman, G., Deftos, L. J., and Banga, A. K. 2000. Transdermal iontophoretic delivery of salmon calcitonin. Int J Pharm **25**: 10–13.

51. Chen, G., Jayawickreme, C., Way, J., Armour, S., Queen, K., Watson, C., Ignar, D., Chen, W., and Kenakin, T. Constitutive receptor systems for drug discovery. 1999. J Pharmacol Toxicol Meth **42**: 199–206.

52. Cheng, J.B., Motola, D.L., Mangelsdorf, D.J., Russell, D.W. 2003. *J. Biol. Chem.* **278**: 38084–38093.

53. Cheng, W.W., Allen, T.M. Targeted delivery of anti-CD19 liposomal doxorubicin in B-cell lymphoma: a comparison of whole monoclonal antibody, Fab' fragments and single chain Fv. 2008. J Control Release **126** (1): 50-58.

54. Cheng, W., Lim, L.-Y. 2008. Comparison of reversible and non reversible aqueoussoluble lipidized conjugates of salmon calcitonin, Mol. Pharm. **5**: 610–621.

55. Cheng, W., Lim, L-Y. 2009. Lipeo-sCT: A novel reversible lipidized salmon calcitonin derivative, its biophysical properties and hypocalcemic activity. Eur J Pharm Sci **37**(2): 151-159.

56. Chesneau, V. *et al.* 2003. Catalytic properties of ADAM19. *J. Biol. Chem.* **278:** 22331–22340.

57. Chestnut, C.H. III, Silverman, S., Andriano, K., et al., for the PROOF Study Group. 2000. A randomized trial of nasal spray salmon calcitonin in postmenopausal women with established osteoporosis: the PROOF study. Am J Med. **109**: 267–276.

58. Chiusaroli, R., Sanjay, A., Henriksen, K., Engsig, M. T., Horne, W. C., Gu, H., and Baron, R. 2003. Dev. Biol. **261**: 537–547.

59. Chong, B., *et al.* 2003. Idiopathic hyperphosphatasia and TNFRSF11B mutations: relationships between phenotype and genotype. *J. Bone. Miner. Res.* **18:** 2095–2104.

60. Christakos, S., Dhawan, P., Liu, Y., Peng, X., Porta, A. 2003. *J. Cell. Biochem.* **88**: 695–705.

61. Coleman, E., Hedin, R., Swann, J., Orloff, D. 2002. A brief history of calcitonin. The Lancet **359** (9309): 885-886.

62. Collin-Osdoby, P., Xuefeng, Y., Zheng, H, Osdoby, P. 2003. RANKL-mediated osteoclast formation from murine RAW 264.7 cells. Methods in Mol Med **80**: 153-165.

63. Copp, D. H., Cameron, E. C., Cheney, B. A., Davidson, A. G. F., and Heinze K. G. 1962. Evidence for calcitonin—A new hormone from the parathyroid that lowers blood calcium. Endocrinology **70**: 638–649.

64. Coleman, P. J., Brashear, K. M., Askew, B. C. et al. 2004. Non peptide αvβ3 antagonists. Part 11: discovery and preclinical evaluation of potent αvβ3 antagonists for the prevention and treatment of osteoporosis. J. Med. Chem. **47**: 4829–4837.

65. Couse, J.F., and Korach, K.S. 1999. ER receptor null mice: what have we learned and where will they lead us? Endocr. Rev. **20:** 358–417.

66. Cummings, S.R., Chapurlat, R.D. 2000. What PROOF proves about calcitonin and clinical trials. Am J Med **109**: 330-331.

67. Cundy, T., *et al.* 2002. A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. *Hum. Mol.Genet.* **11:** 2119–2127.

68. Currey, J.D. 2002. Bones: structure and mechanics. Princeton, N.J.: Princeton University Press: 1-380.

69. Czupalla, C., Mansukoski, H., Riedl, T., Thiel, D., Krause, E., Hoflack, B. 2006. Mol. Cell. Proteomics **5**: 134–143.

70. Dai, X.M., Ryan, G.R., Hapel, A.J., Dominguez, M.G., Russell, R.G., Kapp, S., et al. 2002. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood **99**: 111−20.

71. Darnay, B.G., et al. 1998. Characterization of the intracellular domain of receptor activator of NFkappaB (RANK). Interaction with tumor necrosis

factor receptor-associated factors and activation of NF-kappab and c-Jun N-terminal kinase. J. Biol. Chem. **273**: 20551–20555.

72. Datta, S.R., Brunet, A., Greenberg, M.E. 1999. Cellular survival: a play in three Akts. Genes Dev. **13**: 2905–2927.
73. Day, C. J., Kim, M. S., Simcock, W. E., Stephens, S. R. J., Aitken, C. J., Nicholson, G. C., Morrison, N. A. 2004. J. Cell. Biochem. **91**: 303–315.
74. de Groen, P.C., Lubbe, D.F., Hirsch, L.J., *et al.* 1996. Esophagitis associated with the use of alendronate, N Engl J Med **335**: 1016–1021.
75. Delaissé, J-M., Andersen, T.L., Engsig M.T., Henriksen, K., Troen, T., Blavier, L. 2003. Matrix metalloproteinases (MMP) and Cathepsin K contribute differently to osteoclastic activities. Microscopy research and technique **61**: 504-513.
76. Del Fattore, A., Peruzzi, B., Rucci, N., Recchia, I., Cappariello, A., Longo, M., Fortunati, D., Ballanti, P., Iacobini, M., Luciani, M., Devito, R., Pinto, R., Caniglia, M., Lanino, E., Messina, C., Cesaro, S., Letizia, C., Bianchini, G., Fryssira, H., Grabowski, P., Shaw, N., Bishop, N., Hughes, D., Kapur, R.P., Datta, H.K., Taranta, A., Fornari, R., Migliaccio, S., Teti, A. 2006. J. Med. Genet. **43**: 315–325.
77. DeLuca, H.F. 1988. The vitaminD story: a collaborative effort of basic science and clinical medicine. FASEB J **2**: 224–236.
78. DeLuca, H.F. 2004. *Am. J. Clin. Nutr.* **80**: 1689S–1696S.
79. Dempster, D. W. 2000. The contribution of trabecular architecture to cancellous bone quality. J Bone Miner Res **15**: 20–23.
80. Destaing, O., Saltel, F., Geminard, J.C., Jurdic, P., Bard, F. 2003.Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. Mol Biol Cell **14**: 407–16.

81. De Vernejoul, M. C., Benichou, O. 2001. Human osteopetrosis and other sclerosing disorders: recent genetic developments. Calcif. Tissue Int. **69**: 1–6.

82. Dogru, S. T., Calis, S., and Oner, F. 2000. Oral multiple w/o/w emulsion formulation of a peptide salmon calcitonin: In vitro and in vivo evaluation. J Clin Pharm Ther **25**: 435–443.

83. Dostal, L.A., Toverud, S.U. 1984. Effect of vitamin D3 on duodenal calcium absorption *in vivo* during early development. Am J Physiol **246**: G528–G534.

84. Dougall, W.C., *et al.* 1999. RANK is essential for osteoclast and lymph node development. *Genes. Dev.* **13:** 2412–2424.

85. Drory, O., and Nelson, N. 2006. Physiology **21**: 317-325.

86. Duong, L.T., Lakkakorpi, P.T., Nakamura, I., Machwate, M., Nagy, R.M., Rodan, G.A. 1998. PYK-2 in osteoclasts is an adhesion kinase, localized in the sealing zone, activated by ligation of alpha(v)beta3 integrin, and phosphorylated by src kinase. J. Clin. Invest. **102**:881-892.

87. Ebeling, P.R., et al. 1996. Bone turnover markers and bone density across the menopausal transition. *J. Clin. Endocrinol. Metab.* **81**:3366–3371.

88. Emson, P. C., and Zaidi, M. Further evidence for the origin of circulating calcitonin gene-related peptide in the rat. 1989.J Physiol **412**: 297–308.

89. Engleman, V.W., Nickols, G.A., Ross, F.P., Horton, M.A., Settle, S.L., Ruminski, P.G., Teitelbaum, S.L. 1997. A peptidomimetic antagonist of the $\alpha v\beta 3$ integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. J Clin Invest **99**: 2284–2292.

90. Engsig, M.T., Chen, Q.J., Vu, T.H., Pedersen, A.C., Therkidsen, B., Lund, L.R., Henriksen, K., Lenhard, T., Foged, N.T., Werb, Z., Delaissé, J.M. 2000a. Matrix metalloproteinase 9 and vascular endothelial growth factor are

essential for osteoclast recruitment into developing long bones. J Cell Biol **151**:879 – 889.

91. Engsig, M.T., Henriksen, Foged, N.T., Delaissé, J.M. 2000b. ODF and VEGF induce a stimulation of osteoclast migration which is inhibited by their respective antogonists, OPG and Endostatin. J Bone Miner Res **15**: S278 (abstract).
92. Ettinger, B., Black, D.M., Mitlak, B.H., Knickerbocker, R.K., Nickelsen, T., Genant, H.K., et al. 1999. Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene—results froma 3-year randomized clinical trial. JAMA **282**: 637–45.
93. Evans, R.M. 1988. The steroid and thyroid receptor superfamily. Science **240**: 889–895.
94. Everts, V., Delaissé, J.M., Korper, W., Jansen, D.C., Tigchelaar-Gutter, W., Saftig, P., Beertsen, W. 2002. The bone-lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res **17**:77–90.
95. Everts, V., Korper, W., Hoeben, K.A., Jansen, I.D., Bromme, D., Cleutjens, K.B., Heeneman, S., Peters, C., Saftig, P., Beertsen, W. 1989. J. Bone Miner. Res. **21**: 1399-1408.
96. Faccio, R., Zallone, A., Ross, F.P., Teitelbaum, S.L. 2003. c-Fms and the $\alpha v\beta 3$ integrin collaborate during osteoclast differentiation. J Clin Invest **111**: 749–758.
97. Faccio, R., Takeshita, S., Colalanni, G., Chappel, J., Zallone, A., Teitelbaum, S.L., Ross, F.P. 2007. M-CSF regulates the cytoskeleton via recruitment of a multimeric signaling complex to c-FmsTyr-559/697/721. J Biol Chem **287** (26): 18991-18999.

98. Faccio, R., Teitelbaum, S.L., Fujikawa, K., Chappel, J.C., Zallone, A., Tybulewicz, V.L., Ross, F.P., Swat, W. 2005. Vav3 regulates osteoclast functionand bone mass. Nat Med **11**: 284–290

99. Fata, J.E. *et al.* 2000. The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103:** 41–50.

100. Felix R, Cecchini MG, Fleisch H (1990): Macrophage colony-stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. Endocrinology **127**:2592–2594.

101. Fleisch, H., Russell, R.G., Francis, M.D. 1969. Diphosphonates inhibit hydroxyapatite dissolution in vitro and bone resorption in tissue culture and in vivo. Science **165**: 1262–4.

102. Fleisch, H. 1997. Bisphosphonates: Mechanisms of action and clinical use in osteoporosis–an update. Horm Metab Res **29**: 145– 150.

103. Font, R.G., Garcia, M.L.M., Martinez, J.M.O. 2008. Osteochemonecrosis of the jaws due to bisphosphonate treatments. Update. Med Oral Patol Cir Bucal **13** (5): E318- 24.

104. Foord, S.M. 2002. Receptor classification: post genome. Curr. Opin. Pharmacol. **2:** 561–566.

105. Force, T., Bonventre, J. V., Flannery, M. R., Gorn, A. H., Yamin, M., Goldring, S. R. 1992. A cloned porcine renal calcitonin receptor coupled to adenylyl cyclase and phospholipase C. Am J Physiol **262**: F1110–F1115.

106. Franzoso, G., et al. 1997. Requirement for NFkappaB in osteoclast and B-cell development. Genes. Dev. **11**: 3482–3496.

107. Friedman, J., and Raisz, L.G. 1965. Thyrocalcitonin inhibitor of bone resorption in tissue culture. Science **150**: 1465-1467.

108. Frost, H.M. 1986. Intermediary Organization of the Skeleton. Boca Raton, FL: CRC Press.

109. Fuller, K., Chambers, T.J. 1995. Localisation of mRNA for collagenase in osteocytic, bone surface and chondrocytic cells but not osteoclasts. J Cell Sci **108**: 2221–2230.

110. Fuller, K., Kirstein, B., Chambers, T.J. 2007. Clin. Sci. (Lond) **112**: 567–575.

111. Fuller, K., Owens, J.M., Jagger, C.J., Wilson, A., Moss, R., Chambers, T.J. 1993. Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med **178**:1733–1744.

112. Galibert, L., et al. 1998. The involvement ofmultiple tumor necrosis factor receptor (TNFR)-associated factors in the signaling mechanisms of receptor activator of NF-kappaB, a member of the TNFR superfamily. J. Biol. Chem. **273**: 34120–34127.

113. Gardella, R.J., Jüppner, H. 2000. Interaction of PTH and PTHrP with their receptors. Rev. Endocr. Metab. Dis. **1**: 317-329.

114. Gatti D, Adami S 1999 New bisphosphonates in the treatment of bone diseases. Drugs Aging 15:285–296.

115. Gelb, B.D., Shi, G.P., Chapman, H.A., Desnick, R.J. 1996. Science **273**: 236–1238.

116. Gershengorn, M.C. and Osman, R. 2001. Minireview: Insights into G protein-coupled receptor function using molecular models. Endocrinology **142** (1): 2-10.

117. Gillespie, M.T., Thomas, R.J., Pu, Z.Y., Zhou, H., Martin, T.J., and Findlay, D.M. 1997. Calcitonin receptors, bone sialoprotein and osteopontin are expressed in primary breast cancers. Int. J. Cancer **73**: 812-815.

118. Glantschnig, H., Fisher, J.E., Wesolowski, G., Rodan, G.A., Reszka, A.A.

2003. M-CSF, TNFα and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death and Differentiation **10**: 1165-1177.

119. Goderie-Plomp, H.W., van der Klift, M., de Ronde, W., et al. 2004. Endogenous sex hormones, sex hormone-binding globulin, and the risk of incident vertebral fractures in elderly men and women: The Rotterdam Study. J Clin Endocrinol Metab. **89**: 3261-3269.

120. Goldberg, A.F., Barka, T. 1962. Acid phosphatase activity in human blood cells. Nature **189**: 297.

121. Goldring, S.R., Dayer, D.M., Ausiello, D.A., and Krane, S.M. 1978. A cell strain cultured from porcine kidney increases cyclic AMP content upon exposure to calcitonin or vasopressin. Biochem. Biophys. Res. Commun. **83**: 434-440.

122. Goldring, S.R., Dayer, D.M., Ausiello, D.A., and Krane, S.M. 1978. A cell strain cultured from porcine kidney increases cyclic AMP content upon exposure to calcitonin or vasopressin. Biochem. Biophys. Res. Commun. **83**: 434-440.

123. Goldring, S. R., Gorn, A. H., Yamin, M., Krane, S. M., Wang, J. T. 1992. Characterization of the structural and functional properties of cloned calcitonin receptor cDNAs. Horm Metab Res **25**: 477–480.

124. Gonzalez, D., Ghiringhelli, G., and Mautalen, C. 1986. Acute anti-osteoclastic effect of salmon calcitonin in osteoporotic women. Calcif Tissue Int **38**:71–75.

125. Gonzalez-Suarez, E., et al. 2007. RANK overexpression in transgenic mice with mouse mammary tumor virus promoter-controlled RANK increases proliferation and impairs alveolar differentiation in the mammary epithelia and disrupts lumen formation in cultured epithelial acini. Mol. Cell Biol. **27**: 1442-

1454.

126. Gowen, M., Lazner, F., Dodds, R., Kapadia, R., Field, J., Tavaria, M., Bertonelle, I., Drake, F., Zavarselk, S., Tellis, I., Hertzog, P., Debouck, C., Kola, I. 1999. J Bone Miner Res **14**: 1654–1663.

127. Graham, R., Russell, G. 2007. Bisphosphonates: mode of action and pharmacology. Pediatrics **119**: S150-S162.

128. Graham, R., Russell, G., Espina,B., and Hulley, P. 2006. Bone biology and the pathogenesis of osteoporosis. Curr.Opin. Rheumatol. **18** (suppl.1): S3-S10.

129. Grano, M., Galimi, F., Zambonin, G., Colucci, S., Cottone, E., Zallone, A.Z., Comoglio, P.M. 1996. Proc. Natl. Acad. Sci. USA **93:** 7644–7648.

130. Gray, C., Boyde, A., Jones, S.J. 1996. Bone **18**: 115–123.

131. Green, J.R., Rogers, M.J. 2002. Pharmacological profile of zoledronic acid: a highly potent inhibitor of bone resorption. Drug Dev Res **55**: 210–224.

132. Grey, A.B., Stapleton, J.P., Evans, M.C., Tatnell, M.A., Ames, R.W., Reid, I.R. 1995. The effect of the antiestrogen tamoxifen on bone mineral density in normal late postmenopausal women. Am J Med **99**: 636–41.

133. Gupta, A., Tenenhouse, H.S., Hoag, H.M., Wang, D., Khadeer, M.A., Namba, N., Feng, X., Hruska, K.A. 2001. Bone **29**: 467–476.

134. Gutkind, J. S. 1998. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. J Biol Chem. **273**: 1839–1842.

135. Haddad, J.G.1995. *J. Steroid Biochem. Mol. Biol.* **53**: 579–582.

136. Hadjidakis, D.J., and Androulakis, I.I. 2006. Bone Remodeling. Ann. N.Y. Acad. Sci. **1092**:385-396.

137. Halleen, J.M., Räisänen, S.R., Alatalo, S.L., Väänänen, H.K. 2003. J. Bone

Miner. Res. **18**: 1908–1911.

138. Halleen, J.M., Tiitinen, S.L., Ylipahkala, H., Fagerlund, K.M., Väänänen, H.K. 2006. Clin. Lab. **52**: 499–509.

139. Hamm, H.E. 1998. The many faces of G protein signaling. J Biol Chem 273: 669-672.

140. Han, X., Sterling, H., Chen, Y., Saginario, C., Brown, E.J., Frazier, W.A., Lindberg, F.P., Vignery, A. 2000. CD47, a ligand for the macrophage fusion receptor, participates in macrohage multinucleation. J Biol Chem. **275**:37984–92.

141. Han, Y., Cowin, S.C., Schaffler, M.B., Wein-baum, S. 2004. Mechanotransduction and strain amplification in osteocyte cell processes. Proc Natl Acad Sci USA **101**:16689- 94.

142. Hastewell, J., Lynch, S., Williamson, I., Fox, R., Mackay, M. 1992. Absorption of human calcitonin across the rat colon in vivo. Clin. Sci. **82**: 589–594.

143. Hauge, E.M., Qvesel, D., Eriksen, E.F., et al. 2001. Cancellous bone remodelling occurs in specialized compartments lined by cells expressing osteoblastic markers. Journal of Bone and Mineral Research **16** (9): 1575-1582.

144. Hayden, M.S., & Ghosh, S. 2008. Shared principles in NF-kappaB signaling. Cell **132**: 344–362.

145. Hayman, A.R., Bune, A.J., Bradley, J.R., Rashbass, J., Cox, T.M. 2000. Osteoclastic tartrate-resistant acid phosphatase (Acp5): Its localization to dendritic cells and diverse murine tissues. J. Histochem. Cytochem. **48** (2): 219-227.

146. Hazenberg, J.G., Freeley, M., Foran, E., et al. 2006. Microdamage: a cell transducing mechanism based on rup-tured osteocyte processes. Journal of Biomechanics **39**(11): 2096–2103.

147. Hikita, A., *et al.* 2006. Negative regulation of osteoclastogenesis by ectodomain shedding of receptor activator of NF-kappaB ligand. *J. Biol. Chem.* **281:** 36846–36855.

148. Holtrop, N. E., Raisz, L. J., Simmons, H. A. 1974. The effects of parathyroid hormone, colchicines and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. J Cell Biol **60**: 346–355.

149. Hotokezaka, H., et al. 2002. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J. Biol. Chem. **277**: 47366–47372.

150. Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., Tan, H. L., Elliott, G., Kelley, M. J., Sarosi, I. 1999. Proc. Natl. Acad. Sci. USA **96:** 3540–3545.

151. Hughes, D.E., Dai, A., Tiffee, J.C., Li, H.H., Mundy, G.R., Boyce, B.F. 1996. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF-β. Nature Medicine **2**(10): 1132-1135.

152. Hughes, D. E., Wright, K. R., Uy, H. L., Sasaki, A., Yoneda, T., Roodman, G. D., Mundy, G. R., and Boyce, B. F. 1995 J. Bone Miner. Res. **10**: 1478–1487.

153. Huwyler, R., Born, W., Ohanhaus, E.E., Fischer, J.A. 1979. Plasma kinetics and urinary excretion of exogenous human and salmon calcitonin in man. Am. J. Physiol. **236**: E15–E19.

154. Ignar-Trowbridge, D.M., Teng, C.T., Ross, K.A., Parker, M.G., Korach, K.S., McLachlan, J.A. 1993. Peptide growth factors elicit estrogen receptor-

dependent transcriptional activation at an estrogen-responsive element. Mol Endocrinol **7**: 992–998.

155. Ikeda, T., *et al.* 2001. Determination of three isoforms of the receptor activator of nuclear factor kappa B ligand and their differential expression in bone and thymus. *Endocrinology* **142:** 1419–1426.

156. Ingulli, E., et al. 1997. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. J. Exp. Med. **185**: 2133–2141.

157. Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K.L. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. **4**: 648–657.

158. Inoue, M., Namba, N., Chappel, J., Teitelbaum, S.L., Ross, F.P. 1998. Granulocyte macrophage colony stimulating factor reciprocally regulates _v-associated integrins on murine osteoclast precursors. Mol Endocrinol **12**: 1955–1962.

159. Inoue, D., Shih, C., Galson, D.L., Goldring, S.R., Horne, W.C., Baron, R. 1999. Calcitonin- dependent down-regulation of the mouse C1a calcitonin receptor in cells of the osteoclast lineage involves a transcriptional mechanism. Endocrinology **140**: 1060–1068.

160. Inoue, J., et al. 2000. Tumor necrosis factor receptorassociated factor (TRAF) family: adapter proteins that mediate cytokine signaling. Exp. Cell Res. **254**: 14–24.

161. Insogna, K.L., Sahni, M., Grey, A.B., Tanaka, S., Horne, W.C., Neff, L., Mitnick, M., Levy, J.B., Baron, R. 1997a. Colony-stimulating factor-1 induces cytoskeletal reorganization and c-src-dependent tyrosine phosphorylation of selected cellular proteins in rodent osteoclasts. J Clin Invest **100**: 2476–2485.

162. Insogna, K., Tanaka, S., Neff, L., Horne, W., Levy, J., Baron, R. 1997b. Role of c-Src in cellular events associated with colony-stimulating factor-1- induced spreading in osteoclasts. Mol Reprod Dev **46**:104–108.

163. Inzerillo, A.M., Zaidi, M. 2002. Osteoporosis: Trends and intervention. Mt Sinai J Med **69**(4): 220-231.

164. Iotsova, V., et al. 1997. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat. Med. **3**: 1285–1289.

165. Ishida, N., Hayashi, K., Hoshijima, M., Ogawa, T., Koga, S., Miyatake, Y., Kumegawa, M., Kimura, T., Takeya, T. 2002. J. Biol. Chem. **277**: 41147–41156.

166. Ishibashi, T., Ding, L., Ikenaka, K., Inoue, Y., Miyado, K., Mekada, E., Baba, H. 2004. Tetraspanin protein CD9 is a novel paranodal component regulating paranodal junctional formation. J Neurosci. **24**: 96–102.

167. Ishii, M., Saeki, Y. 2008. Osteoclast cell fusion: mechanisms and molecules. Mod Rheumatol **18**: 220-227.

168. Ito, S. *et al.* 2002. Crystal structure of the extracellular domain of mouse RANK ligand at 2.2-A resolution. *J. Biol. Chem.* **277:** 6631–6636.

169. Johnson, M.R., Polymeropoulos, M.H., Vos, H.L., Ortiz de Luna, R.I., Francomano, C.A. 1996. Genome Res **6**: 1050–1055.

170. Josien, R. *et al.* 1999. TRANCE, a TNF family member, is differentially expressed on T cell subsets and induces cytokine production in dendritic cells. *J. Immunol.* **162:** 2562–2568.

171. Kallio, D. M., Ganat, P. R., and Minkin, C. 1972. Ultrastructural effects of calcitonin on osteoclasts in tissue culture. J Ultrastr Res **39**: 205.

172. Kanis, J.A., Melton, L.J. III, Christiansen, C., et al. 1994. The diagnosis of osteoporosis. J Bone Miner Res **9**: 1137 – 1141.

173. Kanis, J. A., and McCloskey, E. V. 1999. Effect of calcitonin on vertebral and other fractures. Q J Med **92**: 143–149.

174. Katzenellenbogen, B.S., Katzenellenbogen, J.A. 2002. Biomedicine defining the "S" in SERMs. Science **295**: 2380–1.

175. Karin, M., & Lin, A. 2002. NF-kappaB at the crossroads of life and death. Nat. Immunol. **3**: 221–227.

176. Karsdal, M.A., Henriksen, K., Sorensen, M.G., Gram, J., Schaller, S., Dziegiel, M.H., Heegaard, A.M., Christophersen, P., Martin, T.J., Christiansen, C., Bollerslev, J. 2005. Am. J. Pathol. **166**: 467–476.

177. Kartsogiannis, V. *et al.* 1999. Localization of RANKL (receptor activator of NF kappa B ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone* **25:** 525–534.

178. Kelley, T.W., Graham, M.M., Doseff, A.I., Pomerantz, R.W., Lau, S.M., Ostrowski, M.C.,Franke, T.F. and Marsh, C.B. 1999. Macrophage colony-stimulating factorpromotes cell survival through Akt/protein kinase B. J. Biol. Chem. **274**: 26393–26398.

179. Khosla, S., et al. 2004. Relationship of estrogen receptor genotypes to bone mineral density and to rates of bone loss in men. J. Clin. Endocrinol. Metab. **89**: 1808–1816.

180. Kitazawa, R., Kimble, R.B., Vannice, J.L., *et al.* 1994. Interleukin-1 receptor antagonist and tumor necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. J. Clin. Invest. **94:** 2397–2406.

181. Kittur, S. D., Hoppener, J. W. M., Anatonarakis, S. E., Daniels, J. D. J., Meyers, D. A., Maestry, N., Jaansen, M., Korneluk, R. G., Nelkin, B. D., and Kazaziam, H. H. 1985.Proc Natl Acad Sci **83**:5064–5067.

182. Kiviranta, R., Morko, J., Alaralo, S.L., NicAmhlaoibh, Risteli, J., Laitala-Leinonen, Vuorio, E. 2007. Bone **36**: 159-172.

183. Kobayashi, N., et al. 2001. Segregation of TRAF6- mediated signaling pathways clarifies its role in osteoclastogenesis. EMBO J. **20**: 1271–1280.

184. Kobayashi, T., et al. 2003. TRAF6 is a critical factor for dendritic cell maturation and development. Immunity **19**: 353–363.

185. Koffer, A., Tatham, P.E., Gomperts, B.D. 1990. J. Cell Biol. **111**: 919.

186. Koga, T., Inui, M., Inoue, K., Kim, S., Suematsu, A., Kobayashi, E., Iwata, T., Ohnishi, H., Matozaki, T., Kodama, T., Taniguchi, T., Takayanagi, H., Takai, T. 2004 Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis. Nature **428**: 758–763.

187. Kollenkirchen, U., Fox, J., Walters, M.R. 1991. Normocalcemia without hyperparathyroidism in vitamin D-deficient rats. J. Bone Miner Res **6**:273–278.

188. Kong, Y.Y., *et al.* 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature **397:** 315–323.

189. Kong, Y.Y., et al. 1999. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature **402**: 304–309.

190. Kornak, U., Kasper, D., Bosl, M.R., Kaiser, E., Schweizer, M., Schultz, A., Friedrich, W., Delling, G., Jentsch, T.J. 2001. Loss of the CIC-7-chloride channel leads to osteopetrosis in mice and man. Cell **104**: 205-215.

191. Kraenzlin, M. E., Seibel, M. J., Trechsel, U., Boerlin, V., Azria, M., Kraenzlin, C. A., and Haas, H. G. 1996. The effect of intranasal salmon calcitonin on postmenopausal bone turnover as assessed by biochemical markers:

Evidence of maximal effect after 8 weeks of continuous treatment. Calcif Tissue Int **58**: 216–220.

192. Kruse, J.J.C.M., Strootman, E.G., Bart, C.I., Visser, A., Leer, J.-W.H., Wondergem, J. 2002. Radiation-induced changes in gene expression and distribution of atrial natriuretic peptide (ANP) in different anatomical regions of the rat heart. Int. J. Radiat. Biol. **78** (4): 297-304.

193. Kubota, K., Sakikawa, C., Katsumata, M., Nakamura, T., Wakabayashi, K. 2002. J. Bone Miner. Res. **17**: 257–265.

194. Kukita, T., Wada, N., Kukita, A., Kakimoto, T., Sandra, F., Toh, K., Nagata, K., Iijima, T., Horiuchi, M., Matsusaki, H., Hieshima, K., Yoshie, O., Nomiyama, H. 2004. RANKL-induced DC-STAMP is essential for osteoclastogenesis. J Exp Med. **200**: 941–6.

195. Kwai, T., Matsuyama, T., Hosokawa, Y., Makihira, S., Seki, M., Karimbux, N.Y., Goncalves, R.B., Valverde, P., Dibart, S., Li, Y.P., et al. 2006. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. Am. J. Pathol. **169**: 987–998.

196. Kwon, B.S., *et al.* 1998. TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption. *FASEB J.* **12:** 845–854.

197. Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., et al. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell **93**:165–76.

198. Laitala-Leinonen, T., Rinne, R., Saukko, P., Väänänen, H.K, Rinne, A. 2006. Matrix Biol **25**: 149–157.

199. Lakkakorpi, P.T., Nakamura, I., Nagy, R.M., Parsons, J.T., Rodan, G.A., Duong, L.T. 1999. Stable association of PYK-2 and p130 (Cas) in osteoclasts and their co-localization in the sealing zone. J. Biol. Chem. **274**: 4900-4907.

200. Lakkakorpi, P.T., and Väänänen, H.K. 1991. J. Bone Miner. Res. **6**:817-826.

201. Lam, J. *et al.* 2001. Crystal structure of the TRANCE/RANKL cytokine reveals determinants of receptor-ligand specificity. *J. Clin. Invest.* **108:** 971–979.

202. Lane, N.E., Kelman, A. 2003. A review of anabolic therapies for osteoporosis. Arthritis Res Ther **5**(5): 214-222.

203. Lane, N.E., Yao, W., Nakamura, M.C., Humphrey, M.B., Kimmel, D., Huang, X., Sheppard, D., Ross, F.P., Teitelbaum, S.L. 2005. Mice lacking the integrin beta5 subunit have accelerated osteoclast maturation and increased activity in the estrogen-deficient state. J Bone Miner Res **20**: 58–66.

204. Lee, K., Jessop, H., Suswillo, R., Zaman, G., and Lanyon, L. 2003. Endocrinology: bone adaptation requires oestrogen receptor-alpha. *Nature.* **424**: 389.

205. Lee, Y. H., Perry, B. A., Sutyak, J. P., Stern, W., and Sinko, P. J. 2000. Regional differences in intestinal spreading and pH recovery and impact on salmon calcitonin absorption in dogs. Pharm Res 17:284–290.

206. Lee, Z.H., et al. 2000. Activation of c-JunN-terminal kinase and activator protein 1 by receptor activator of nuclear factor kappa B. Mol. Pharmacol. **58**: 1536–1545.

207. Leibbrandt, A., Penninger, J.M. 2008. RANK/RANKL: Regulators of immune responses and bone physiology. Ann. N.Y. Acad. Sci. **1143**: 123-150.

208. Li, J. *et al.*, 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. *Proc. Natl. Acad. Sci. USA* **97:** 1566–1571.

209. Lim, S., Joung, H., Shin, C.S., et al. 2004. Body composition changes with age have gender-specific impacts on bone mineral density. Bone **35**: 792-798.

210. Lindsay R, Hart DM, Forrest C, Baird C. 1980. Prevention of spinal osteoporosis in oophorectomised women. Lancet **ii**: 1151–3.

211. Linsuwanont-Santiwong, B., Takagi, Y., Ohya, K., Shimokawa, H. 2006. J. Bone Miner. Metab. **24**: 447–453.

212. Ljusberg, J., Wang, Y., Lång, P., Norgård, M., Dodds, R., Hultenby, K., Ek-Rylander, B., Andersson, G. 2005. J Biol Chem **280**: 28370–28381.

213. Locksley, R.M., Killeen, N., Lenardo, M.J. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104:** 487–501.

214. Lomaga, M.A., et al. 1999. TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes. Dev. **13**: 1015–1024.

215. Loser, K. *et al.* 2006. Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells. *Nat. Med.* **12:** 1372–1379.

216. Lotinun, S., Evans, G.L., Turner, R.T., Oursler, M.J. 2005. J. Bone Miner. Res. **20**: 644–652.

217. Lou, H. and Gagel, R. F. 1998. Alternative RNA processing—Its role in regulating expression of calcitonin/calcitonin gene-related peptide. J Endocrinol **156**: 401–405.

218. Lou, H., Cote, G. J., and Gagel, R. F. 1994. The calcitonin exon and its flanking intronic sequences are sufficient for the regulation of human calcitonin/ calcitonin gene-related peptide alternative RNA splicing. Mol Endocrinol **8**:1618–1626.

219. Lou, H., Gagel, R. F., and Berget, S. M. 1996. An intron enhancer recognized by splicing factors activates polyadenylation. Genes Dev **10**:208–219.

220. Lorget, F., Kamel, S., Mentaverri, R., Wattel, A., Naassila, M., Maamer, M., Brazier, M. 2000. Biochem. Biophys. Res. Commun. **268**: 899–903.

221. Lynch, C.C. *et al.* 2005. MMP-7 promotes prostate cancer-induced osteolysis via the solubilization of RANKL. *Can. Cell* **7:** 485–496.

222. Manolagas, S.C.1995. Role of cytokines in bone resorption. Bone **17:** 63S–67S.

223. Manolagas, S.C., Kousteni, S., Jilka, R.L. 2002. Sex steroids and bone. Recent Prog. Horm. Res. **57:** 385–409.

224. Mansoor, S., Youn, Y.S., Lee, K.C. 2005. Oral delivery of mono-PEGylated sCT (Lys18) in Rats: Regional difference in stability and hypocalcemic effect. Pharm Dev Tech **10**: 389-396.

225. Mao, D., et al. 2006. PLCgamma2 regulates osteoclastogenesis via its interaction with ITAM proteins and GAB2. J. Clin. Invest. **116**: 2869–2879.

226. Marchisio, P.C., Bergui, L., Corbascio, G.C., Cremona, O., D'Urso, N., Schena, T.L., Caligaris-Cappio, F. 1988. Vinculin, talin, and integrins are localized at specific adhesion sites of malignant B-lymphocytes. Blood **72**: 830-833.

227. Maricic, M. 2007. New and emerging treatments for osteoporosis. Curr Opin Rheumatol **19**: 364-369.

228. Martin, T.J. 2000. Bisphosphonates- mechanisms of action. Aust Prescr **23**: 130-132.

229. Martin, T.J., and Seeman, E. 2008. Bone Remodelling: its local regulation and the emergence of bone fragility. Best Practice & Research Clinical Endocrinology & Metabolism **22** (5): 701-722.

230. Martin, T.J. and Seeman, E. 2007. New mechanisms and targets in the treatment of bone fragility. Clin Sci **112**: 77-91.

231. Marx, R.E. 2003. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic, J Oral Maxillofac Surg **61**: 1115–1118.

232. Mashiba, T., Hirano, T., Turner, C.H., *et al.* 2001. Suppressed bone turnover by bisphosphonates increases microdamage accumulation and reduces some biomechanical properties in dog rib. J Bone Miner Res **5**: 613.

233. Matuso, K, Irie, N. 2008. Osteoclast-osteoblast communication. Archives of Biochemistry and Biophysics **473**: 201-209.

234. Mauck, R.L., Wang, C.C-B., Oswald, E.S., Ateshian, G., Hung, C.T. 2003. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis and Cartilage **11** (12): 879-890.

235. Mauck, K.F., Clarke, B.L. 2006. Diagnosis, screening, prevention, and treatment of osteoporosis. *Mayo Clin Proc.* **81**(5): 662-672.

236. McHugh, K.P., Hodivala-Dilke, K., Zheng, M.H., Namba, N., Lam, J., Novack, D., Feng, X., Ross, F.P., Hynes, R.O., Teitelbaum, S.L. 2000. Mice lacking β3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest **105**: 433–440

237. Meng, F., Lowell, C.A. 1998. A b1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration, EMBO J. **17**: 4391–4403.

238. Meiyanto, E., Hoshijima, M., Ogawa, T., Ishida, I., Takeya, T. 2001. Bioch Biophys Res Comm **282**: 278-283.

239. Monla, Y. T., Peleg, S., Gagel, R. F., and Monla, Y. T. 1995.Cell type-specific regulation of transcription by cyclic adenosine 3',5'-monophosphate-responsive elements within the calcitonin promoter. Mol Endocrinol 9:784–793.

240. Moonga, B. S., Moss, D. W., Patchell, A., Zaidi, M. 1990. Intracellular regulation of enzyme secretion from rat osteoclasts and evidence for a functional role in bone resorption. J Physiol **429**: 29–45.

241. Moore, E. E., Kuestner, R. E., Stroop, S. D., Grant, F. J., Matthewes, S. L., Brady, C. L., Sexton, P. M., and Findlay,D.M. 1995. *Mol. Endocrinol.* **9**: 959–968.

242. Muallem, S., Kwiatkowska, K., Xu, X., Yin, H.L. 1995. J. Cell Biol. **128**: 589.

243. Mulari, M., Zhao, H., Lakkakorpi, P, Väänänen, H.K. 2003. Ruffled border membrane in bone resorbing osteoclasts has subdomains for secretion and degraded matrix uptake. Traffic **4**: 113-125.

244. Naito, A., et al. 1999. Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. Genes. Cells. **4**: 353– 362.

245. Nakamura, I., et al. 1995. Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption. FEBS Lett. **361**: 79–84.

246. Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K., Harada, Y., Azuma, Y., Krust, A., Yamamoto, Y., Nishina, H., Takeda, S., Takayanagi, H., Metzger, D., Kanno, J., Takaoka, K., Martin, T.J., Chambon, P., Kato, S. 2007. Cell **130**: 811–823

247. Nakamura, A., Ly, C., Cipetic', M., Sims, N.A., Vieusseux, J., Kartsogiannis, V., Bouralexis, S., Saleh, H., Zhou, H., Price, J.T., Martin, T.J., Ng, K.W., Gillespie, M.T., Quinn, J.M. 2007. Bone **40**: 305–315.

248. Nalla, R.K., Kruzic, J.J., Kinney, J.H., Ritchie, R.O. 2004. Effect of aging on the toughness of human cortical bone: evaluation by R-curves. Bone **35**: 1240-6.

249. Naro, F., Perez, M., Migliaccio, S., Gelson, D. L., Orosti, P., Teti, A., Goldring, S. R. 1998. Phospholipase D and protein kinase C isoenzyme-dependent signal transduction pathways activated by the calcitonin receptor. Endocrinology **139**: 3241–3248.

250. National Osteoporosis Foundation. 2002. America's bone health: the state of osteoporosis and low bone mass in our nation. Washington, DC: National Osteoporosis Foundation.

251. Nelson, C.M., Pirone, D.M., Tan, J.L., Chen, C.S. 2004. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol Biol Cell **15** (6): 2943-2953.

252. Nicholson, G. C., Moseley, J. M., Sexton, P. M., Mendelsohn, F. A. O., and Martin, T. J. 1986. Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization. J Clin Invest **78**: 355–360.

253. Nicolin, V., Ponti, C., Narducci, P., Grill, V., Bortul, R., Zweyer, M., Vaccarezza, M., Zauli, G. 2005. Different levels of the neuronal nitric oxide synthase isoform modulate the rate of osteoclastic differentiation of TIB-71 and CRL-2278 RAW 264.7 murine cell clones. The Anatomical Record Part A **283A**: 945-954.

254. Nielsen, R.H., Karsdal, M.A., Sorensen, M.G., Dziegiel, M.H., Henriksen, K. 2007. Biochem. Biophys. Res. Commun. **360**: 834–839.

255. Niyibizi, C., Eyre, D.R. 1994. Structural characteristics of crosslinking sites in type V collagen of b[redacted] chain specificities and heterotypic links to type I collagen. Eur J Biochem **224**:943–950.

256. Nussenzveig, D. R., Thaw, C. N., and Gershengorn, M. C. 1994. *J. Biol. Chem.* **269**:28123–28129.

257. O'Donnell, S., Cranney, A., Wells, G.A., Adachi, J., Reginster, J.Y. 2006. Strontium ranelate for preventing and treating postmenopausal osteoporosis. Cochrane Database of Systematic Reviews Issue 4.

258. Ohyama, Y., Yamasaki, T. 2004. *Front. Biosci.* **9**: 3007–3018.

259. Orcell, P., Tajima, H., Murayama, Y., Fujita, T., Krane, S. M., Ogata, E., Goldring, S. R., and Nishimoto, I. 2000. Multiple domains interacting with Gs in the porcine calcitonin receptor. Mol Endocrinol **14**: 170–182.

260. Osteoporosis Canada. 2009. http://www.osteoporosis.ca/index.php/ci_id/5526/la_id/1.htm accessed on June 16, 2009.

261. O'Sullivan, S., Naot, D., Callon, K., Porteous, F., Horne, A., Wattie, D., Watson, M., Cornish, J., Browett, P., Grey, A. 2007. J. Bone Miner. Res. **22**: 1679–168.

262. Oursler, M.J., *et al.* 1991. Modulation of transforming growth factor-β production normal human osteoblast-like cells by 17 β -estradiol and parathyroid hormone. Endocrinology **129**: 3313-3320.

263. Overgaard, K., Lindsay, R., and Christiansen, C. 1995. Patient responsiveness to calcitonin salmon nasal spray: A subanalysis of a 2-year study. Clin. Ther. **17**: 680–685.

264. Owen, T.A., Aronow, M.S., Barone, L.M., Bettencourt, B., Stein, G.S., Lian, J.B. 1991. *Endocrinology* **128**: 1496–1504.

265. Pacifici, R., Carano, A., Santoro, S.A., *et al.* 1991. Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells. J. Clin. Invest. **87:** 221–228.

266. Palokangas, H., Mulari, M., Väänänen, H.K. 1997. Endocytic pathway from the basal plasma membrane to the ruffled border membrane in bone-resorbing osteoclasts. J. Cell. Sci. **110**: 1767-1780.

267. Parfitt, A.M. 1994. Osteonal and hemi-osteonal remodelling: The spatial and temporal framework for signal traffic in adult human bone. J Cell Biochem **55**: 273-286.

268. Parfitt, A.M. 1996. Skeletal heterogeneity and the purposes of bone remodelling: implications for the understanding of osteoporosis. In Marcus R, Feldman D, and Kelsey J (eds.). Osteoporosis. San Diego, CA: Academic Press: 315-339.

269. Parfitt, A.M. 2001. The bone remodelling compartment: a circulatory function for bone lining cells. Journal of Bone and Mineral Research **16**(9) 1583-1585.

270. Parfitt, A.M., Villanueva, A.R., Foldes, J., and Rao, D.S. 1995. Relations between histologic indices of bone formation: implications for the pathogenesis of spinal osteoporosis. *J. Bone Miner. Res.***10**: 466–473.

271. Patton, J. S. 2000. Pulmonary delivery of drugs for bone disorders. Adv Drug Deliv Rev **42**: 239–245.

272.

273. Peleg, S., Abruzzese, R. V., Cooper, C. W., Gagel, R. F. 1993.Downregulation of calcitonin gene transcription by vitamin D requires two widely separated enhancer sequences. Mol Endocrinol **7**: 999–1008.

274. Pfeilschifter, J., Mundy, G.R. 1987. Proc. Natl. Acad. Sci. USA **84**: 2024–2028.

275. Pondel, M. 2000. Calcitonin and calcitonin receptors: bone and beyond.

276. Potter, C.J., Pedraza, L.G. and Xu, T. 2002. Akt regulates growth by directly phosphorylating Tsc2. Nat. Cell Biol. 4: 658–665.

277. Qi, L. J., Leung, A. T., Xiong, Y., Marx, K. A., and Abou-Samra, A. B. 1997. Biochemistry **36**: 12442–12448.

278. Qiu, S., Rao, D.S., Fyhrie, D.P., Palnitkar, S., Parfitt, A.M. 2005. The morphological association between microcracks and osteocyte lacunae in human cortical bone. Bone **37**: 10-5.

279. Raisz, L.G. 2005. Pathogenesis of osteoporosis: concepts, conflicts and prospects. Journal of Clinical Investigation **112** (12): 3318-3325.

280. Ralph, P., and Nakoinz, I. 1977. Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: Enhancement by PPD and LPS. J. Immunol. **119:** 950–954.

281. Raval-Pandya, M., Porta, A.R., Christakos, S. 1998. In: M.F. Holick, Editor, *Vitamin D: Physiology, Molecular Biology, and Clinical Applications*, Humana Press Inc., Totowa, NJ: 163–173.

282. Reid, I.R., Schooler, B.A., Hannon, S., Ibbertson, H.K. 1986. The acute biochemical effects of four proprietary calcium supplements. Aust N Z J Med **16**: 193–7.

283. Reid, I.R., Eastell, R., Fogelman, I., Adachi, J.D., Rosen, A., Netelenbos, C., et al. 2004. A comparison of the effects of raloxifene and conjugated equine

estrogen on bone and lipids in healthy postmenopausal women. Arch Int Med **164**: 871–9.

284. Reid, I.R., Mason, B., Horne, A., Ames, R., Reid, H.E., Bava, U., et al. 2006. Randomized controlled trial of calcium in healthy older women. Am J Med **119**: 777–85.

285. Reid, I.R., Ames, R., Mason, B., Reid, H.E., Bacon, C.J., Bolland, M.J., et al. 2008. Randomized controlled trial of calcium supplementation in healthy, non-osteoporotic, older men. Arch Int Med **168** (20): 2276-82.

286. Reszka, A.A., Halasy-Nagy, J., Rodan, G.A. 2001. Nitrogen-bisphosphonates block retinoblastoma phosphorylation and cell growth by inhibiting the cholesterol biosynthetic pathway in a keratinocyte model for esophageal irritation. Mol Pharmacol **59**: 193–202.

287. Rico, H., Hernandez, E. R., Revilla, M. 1992. Salmon calcitonin reduces vertebral fracture rate in post-menopausal crush fracture syndrome. J Bone Miner Res **16**:131–138.

288. Riggs, B.L., Wahner, H.W. 1988. Bone densitometry and clinical decision-making in osteoporosis [editorial]. Ann Intern Med **108**: 293 – 295.

289. Rogers, M.J., Watts, D.J., Russell, R.G.G., et al. 1994. Inhibitory effects of bisphosphonates on growth of amoebae of the cellular slime mold Dictyostelium discoideum. J Bone Miner Res. **9**: 1029–1039.

290. Rogers, M.J., Xiong, X., Brown, R.J., Watts, D.J., Russell, R.G., Bayless, A.V., Ebetino, F.H. 1995. Structure-activity relationships of new heterocycle-containing bisphosphonates as inhibitors of bone resorption and as inhibitors of growth of Dictyostelium discoideum amoebae. Mol Pharmacol **47**: 398–402.

291. Rossouw, J.E., Anderson, G.L., Prentice, R.L., LaCroix, A.Z., Kooperberg, C.,

Stefanick, M.L., et al. 2002. Risks and benefits of estrogen plus progestin in healthy postmenopausal women - Principal results from the Women's Health Initiative randomized controlled trial. JAMA **288**: 321–33.

292. Rotello, R.J., Lieberman, R.C., Purchio, A.F., Gerschenson, L.E. 1991. Coordinated regulation of apoptosis and cell proliferation by transforming growth factor β1 in cultured uterine epithelial cells. Proc. Natl. Acad. Sci. USA **88**: 3412-3415.

293. Rubin MR, Rundek T, McMahon DJ, Lee H-S, Sacco RL, Silverberg SJ. 2007. Carotid artery plaque thickness is associated with increased serum calcium levels: the Northern Manhattan study. Atherosclerosis **194**: 426–32.

294. Ruocco, M.G., et al. 2005. I{kappa}B kinase (IKK){beta}, but not IKK{alpha}, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. J. Exp. Med. **201**: 1677–1687.

295. Russell, R.G., Rogers, M.J., Frith, J.C., Luckman, S.P., Coxon, F.P., Benford, H.L., Croucher, P.I., Shipman, C., Fleisch, H.A. 1999. The pharmacology of bisphosphonates and new insights into their mechanisms of action. J Bone Miner Res **14** (S2): 53–65.

296. Russo, D., Miranda, I., Ruocco, C., Battaglia, Y., Buonanno, E., Manzi, S., et al. 2007. The progression of coronary artery calcification in predialysis patients on calcium carbonate or sevelamer. Kidney Int **72**:1255–61.

297. Ryu, J., Kim, H.J., Chang, E.J., Huang, H., Banno, Y., Kim, H.H. 2006. EMBO J. **25**: 5840–5851.

298. Saftig, P., Hunziker, E., Wehmeyer, O., Jones, S., Boyde, A., Rommerskirch, W., Moritz, J.D., Schu, P., von Figura, K.1998. Proc. Natl. Acad. Sci. USA **95**: 13453– 13458.

299. Saginario, C., Qian, H.Y., Vignery, A. 1995. Identification of an inducible

surface molecule specific to fusing macrophages. Proc Natl Acad Sci USA. **92**:12210–4.

300. Saginario, C., Sterling, H., Beckers, C., Kobayashi, R., Solimena, M., Ullu, E., Vignery, A. 1998. MFR, a putative receptor mediating the fusion of macrophages. Mol Cell Biol. **18**: 6213–23.

301. Salo, J., Lehenkari, P. Removal of osteoclast bone resorption products by transcytosis. 1997. Science **276** (5310): 270-274.

302. Samura, A., Wada, S., Suda, S., Iitaka, M., Katayama, S. 2000. Calcitonin receptor regulation and responsiveness to calcitonin in human osteoclast-like cells prepared in vitro using receptor activator of nuclear factor kappaB ligand and macrophage colony-stimulating factor. Endocrinology **141**: 3774–3782.

303. Sato, T., del Carmen, O.M., Hou, P., Heegaard, A.M., Kumegawa, M., Foged, N.T., Delaissé, J.M. 1997. Identification of the membrane-type matrix metalloproteinaseMT1-MMP in osteoclasts.J Cell Sci **110:**589–596.

304. Schaller, S., Henriksen, K., Sviegaaret, C., et al. 2004. The chloride channel inhibitor NS3736 prevents bone resorption in ovariectomized rats without changing bone formation. J. Bone Miner. Res. **19**: 1144–1153.

305. Schechter, B., Silberman, R., Arnon, R., and Wilchek, M. 1990. Tissue distribution of avidin and steptavidin injected to mice. Effect of avidin, carbohydrate, streptavidin truncation and exogenous biotin. Eur. J. Biochem. **189**: 327-331.

306. Schlesinger, P.H., Blair, H.C., Teitelbaum, S.L., Edwards, J.C. 1997. Characterization of the osteoclast ruffled border chloride channel and its role in bone resorption. J Biol Chem **272**:18636 –18643.

307. Schwartzberg, P.L., Xing, L., Hoffmann, O., Lowell, C.A., Garrett, L., Boyce, B.F., Varmus, H.E. 1997. Rescue of osteoclast function by transgenic

expression of kinase-deficient Src in src-/- mutant mice. Genes Dev **11**: 2835–2844.

308. Seeman, E., and Delmas, P.D. 2006. Bone Quality – The material and structural basis of bone strength and fragility. N Engl J Med **354**: 2250-61.
309. Selander, K. S., Monkkonen, J., Karhukorpi, E. K., Harkonen, P., Hannuniemi, R., and Vaananen, H. K. 1996. Mol. Pharmacol. **50**: 1127–1138.
310. Shadduck, R., Waheed, A., Mangan, K., and Rosenfeld, C. 1993. Preparation of a monoclonal antibody directed against the receptor for murine colony-stimulating factor-1. Exp. Hematol. **21**: 515–520.
311. Shen, W-C., Louie, S.G. 1999. Immunology for pharmacy students. CRC Press.
312. Shimizu, H., Sakamoto, M., Sakamoto, S. 1990. Bone resorption by isolated osteoclasts in living versus devitalized bone: differences in mode and extent and the effects of human recombinant tissue inhibitor of metalloproteinases. J Bone Miner Res **5**:411– 418.
313. Shinki, T., Ueno, Y., DeLuca, H. F., Suda, T. 1999. Calcitonin is a major regulator for the expression of renal 25-hydroxyvitamin D3-1-alpha-hydroxylase gene in normocalcemic rats. Proc Natl Acad Sci **96**: 8253–8258.
314. Shipley, J.M., Wesselschmidt, R.L., Kobayashi, D.K., Ley, T.J., Shapiro, S.D. 1996. Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. Proc Natl Acad Sci USA **93**: 3942–3946.
315. Shuvaev, V.V., Dziubla, T., Wiewrodt, R., Muzykanton, V.R. 2004. Streptavidin-biotin crosslinking of therapeutic enzymes with carrier antibodies: nanoconjugates for protection against endothelial oxidative stress. Methods Mol Biol **283**: 3-19.

316. Shyu, J-F., Inoue, D., Baron, R., Horne, W.C. 1996. The deletion of 14 amino acids in the seventh transmembrane domain of a naturally occurring calcitonin receptor isoform alters ligand binding and selectively abolishes coupling to phoshpholipase C. J. Biol. Chem. **271** (49): 31127-31134.

317. Siegel, R.M. *et al.* 2000. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science* **288:** 2354–2357.

318. Sigurdsson, G., Aspelund, T., Chang, M., et al. 2006. Increasing sex difference in bone strength in old age: The Age, Gene/Environment Susceptibility-Reykjavik study (AGESREYKJAVIK). Bone **39**: 644-651.

319. Silverman, S.L., Mathisen, A., Gallwitz, W. 2006. Effect of parathyroid hormone (1–84) in reducing new vertebral fractures regardless of baseline vertebral fracture status in patients with a wide range of clinical risk factors. J Bone Miner Res **21**:S173.

320. Sims, N.A., et al. 2002. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone.* **30**: 18–25.

321. Simonet, W.S. *et al.* 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89:** 309–319.

322. Sobacchi, C., et al. 2007. Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. Nat. Genet. **39**: 960–962.

323. Spang, A. 2004. Vesicular transport: a close collaboration of Rabs and effectors. Curr. Biol. **14**: R33-R34.

324. Srivastava, S., Weitzmann, M.N., Kimble, R.B. *et al.* 1998. Pacifici RER blocks M-CSF gene expression and osteoclast formation by regulating phosphorylation of Egr-1 and its interaction with Sp-1. J. Clin. Invest. **15:** 1850–1859.

325. SrivasKodama, T., and Taniguchi, T. (2002) Dev. Cell. 31999. ER decreases TNF gene expression by blocking JNK activity and the resulting production of c-Jun and JunD. J. Clin. Invest. **104:** 503–513.

326. Suda, T., Takahashi, N., Martin, T.J. 1992. Modulation of osteoclast differentiation. Endocr Rev **13**:66–80.

327. Tachibana, I., Hemler, M.E. 1999. Role of transmembrane 4 superfamily (TM4SF) proteins CD9 and CD81 in muscle cell fusion and myotube maintenance. J Cell Biol. **146**: 893–904.

328. Takayanagi, H., et al. 2000. T-cell-mediated regulation of osteoclastogenesis by signalling cross - talk between RANKL and IFN-gamma. Nature **408**: 600–605.

329. Takayanagi, H., et al. 2002.RANKLmaintains bone homeostasis through c-Fos-dependent induction of interferon-beta. Nature **416**: 744–749.

330. Takayanagi, H., et al. 2002. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev. Cell. **3**: 889–901.

331. Takayanagi, H., 2007. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. Nat. Rev. Immunol. **7**: 292–304.

332. Takeshita, S., et al. 2002. SHIP-deficient mice are severely osteoporotic due to increased numbers of hyper-resorptive osteoclasts. Nat. Med. **8**: 943–949.

333. Tan, K.B., *et al.* 1997. Characterization of a novel TNF-like ligand and recently described TNF ligand and TNF receptor superfamily genes and their constitutive and inducible expression in hematopoietic and non-hematopoietic cells. *Gene.* **204:** 35–46.

334. Tanaka, S., Amling, L., Neff, L., Peymann, A., Uhlmann, E., Levy, J.B., Baron, R. 1996. C-Cb1 is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature **383**: 528-531.

335. Taylor, D. 1997. Bone maintenance and remodeling: a control system based on fatigue damage. Journal of Orthopaedic Research **15**(4): 601–606.

336. Tee, A.R., Fingar, D.C., Manning, B.D., Kwiatkowski, D.J., Cantley, L.C. and Blenis, J. 2002. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc. Natl. Acad. Sci. USA **99**: 13571–13576.

337. Teitelbaum, S.L. 2000. Osteoclasts, integrins, and osteoporosis. J Bone Miner Metab **18**: 344-349.

338. Teitelbaum, S.L., Ross, F.P. 2003. Genetic regulation of osteoclast development and function. Nat Rev Genet. **4**: 638–49.

339. Tezuka, K., Nemoto, K., Tezuka, Y., Sato, T., Ikeda, Y., Kobori, M., Kawashima, H., Eguchi, H., Hakeda, Y., Kumegawa, M. 1994. Identification of matrix metalloproteinase-9 in rabbit osteoclasts. J Biol Chem **269**:15006 – 15009.

340. The Writing Group for the ISCD Position Development Conference. 2004. Diagnosis of osteoporosis in men, premenopausal women and children. J Clin Densitom **7**:17-26

341. Troen, B.R. 2006. Ann. NY Acad. Sci. **1068**: 165-172.

342. Tsai, M., O'Malley, B.W. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem **63**: 451–486.

343. Tsuda, E. *et al.* 1997. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. *Biochem. Biophys. Res. Commun.* **234:** 137–142.

344. Tukkanen, J. and Väänänen, H.K. 1986. Omerprazole, a specific inhibitor of H+K+-ATPase inhibits bone resorption in vitro. Calcif. Tissue Int. **38**: 123-125.

345. Tuysuz, B., Mercimek, S., Ungur, S., and Deniz, M. 1999. Calcitonin treatment in osteoectasia with hyperphosphatasia (juvenile Paget's disease): Radiographic changes after treatment. Pediatr Radiol **29**:838–841.

346. Ulrich, C.D., Holtmann, M., Miller, L.J. 1998. Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein coupled receptors. Gastroentoerology **114**: 382–397.

347. US Department of Health and Human Services. 2004. Bone health and Osteoporosis: A report of the Surgeon General. Available at: www.surgeongeneral.gov/library/bonehealth. Accessed on Feb.24, 2009.

348. U.S. Preventive Services Task Force. Screening for osteoporosis in postmenopausal women: recommendations and rationale. Rockville, Md.: Agency for Healthcare Research and Quality; September 2002. http://www.ahrq.gov/clinic/3rduspstf/osteoporosis/osteorr.htm. Accessed on April 23, 2009.

349. Väänänen, H.K. 2005. Mechanism of osteoclast mediated bone resorption – rationale for the design of new therapeutics. Advanced Drug Delivery Reviews **57**: 959-971.

350. Väänänen, H.K., and Horton, M. 1995. The osteoclast clear zone is a specialized cell-extracellular matrix adhesion structure. J. Cell Sci. **108**: 2729-2732.

351. Väänänen, H.K., Karhukopi, E.K., Sundquist, K., Wallmark, B., Roininen, I., Hentunen, T., Tuukanen, J., Lakkakorpi, P. 1990. Evidence for the presence of a proton pump of the vacuolar H+-ATPase type in the ruffled vorders of osteolasts. J. Cell. Biol. **111**: 1305-1311.

352. Väänänen, H.K., and Laitala-Leinonen, T. 2008. Osteoclast lineage and function. Archives of Biochemistry and Biophysics **473**: 132-138.

353. Vääräniemi, J., Halleen, J., Kaarlonen, K., Ylipahkala, H., Alatalo, S., Andersson, G., Kaija, H., Vihko, P., Väänänen, H.K. 2004. J. Bone Miner. Res. **19**: 1432–1440.

354. van Cromphaut, S.J., Dewerchin, M., Hoenderop, J.G., Stockmans, I., Van Herck, E., Kato, S., Bindels, R.J., Collen, D., Carmeliet, P., Bouillon, R., Carmeliet, G. 2001. *Proc. Natl. Acad. Sci. USA* **98**: 13324–1332.

355. van der Eerden, B.C., Hoenderop, JG., de Vries, T.J., Schoenmaker, T., Buurman, C.J., Uitterlinden, A.G., Pols, H.A., Bindels, R.J., van Leeuwen, J.P. 2005. Proc. Natl. Acad. Sci. USA **102**: 17507–17512.

356. van der Klift, M., de Laet, C.D., Pols, H.A. 2005. Assessment of fracture risk: Who should be treated for osteoporosis? Best Pract Res Clin Rheumatol. **19**: 937-950.

357. van Pottelbergh, I., Goemaere, S., Zmierczak, H., and Kaufman, J.M. 2004. Perturbed sex steroid status in men with idiopathic osteoporosis and their sons. J. Clin. Endocrinol. Metab. **89**:4949–4953.

358. Verborgt, O., Gibson, G.J., and Schaffler, M.B. 2000. Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue *in vivo*. Journal of Bone and Mineral Research **15**(1): 60–67.

359. Vignery, A. 2005. Macrophage fusion: are somatic and cancer cells possible partners? Trends Cell Biol. **15**:188–193.

360. Viguet-Carrin, S., Garnero, P., Delmas, P.D. 2006. The role of collagen in bone strength. Osteoporos Int. **17**: 319-36.

361. Visentin, L., Dodds, R. A., Valente, M., et al. 2000. A selective inhibitor of the osteoclastic V-H+-ATPase prevents bone loss in both thyroparathyroidechomized and ovariectomized rats. J. Clin. Invest. **106**: 177–179.

362. Vitale, M.L., Rodriguez del Castillo, A., Tchakarov, L., and Trifaro, J.M. 1991. J. Cell Biol. **113:** 1057.

363. Wada, S., Martin, T.J., Findlay, D.M.1995. Homologous regulation of the calcitonin receptor in mouse osteoclast-like cells and human breast cancer T47D cells. Endocrinology **136**:2611–2621.

364. Wada, S., Udagawa, N., Akatsu, T., Nagata, N., Martin, T.J., Findlay, D.M. 1997. Regulation by calcitonin and glucocorticoids of calcitonin receptor gene expression in mouse osteoclasts. Endocrinology **138**: 521–529.

365. Wada, T., et al. 2005. The molecular scaffold Gab2 is a crucial component of RANK signaling and osteoclastogenesis. Nat. Med. **11**: 394– 399.

366. Wainwright, S.A., Biggs, W.D., Currey, J.D., Gosline, J.M. 1982. Mechanical design in organisms. Princeton, N.J.: Princeton University Press: 1-436.

367. Wakeyama, H., Akiyama, T., Takahashi, K., Amano, H., Kadono, Y., Nakamura, M., Oshima, Y., Itabe, H., Nakayama, K.I., Nakayama, K., Nakamura, K., Tanaka, S. 2007. J.Bone Miner. Res. **22**: 1631–1639.

368. Walker, D.G. 1973. Clin. Orthop. Relat. Res. **97**: 158-174. R.H.

369. Warshawsky, H., Goltzman, D., Rouleau, M.F., and Bergeron, J.M. 1980. Direct in vivo demonstration by radioautography of specific binding sites for calcitonin in skeletal and renal tissues of the rat. J. Cell. Biol. **85**: 682-694.

370. Wasserman, C.A. Smith, M.E. Brindak, N. De Talamoni, C.S. Fullmer, J.T. Penniston and R. Kumar, *Gastroenterology* **102** (1992), pp. 686-694.

371. Weinstein, R.S., Underwood, J.L., Hutson, M.S., DeLuca, H.F. 1984. Bone histomorphometry in vitamin D-deficient rats infused with calcium and phosphorus. *Am J Physiol* **246**: E499-E505.

372. Weitzmann, M.N., Roggia, C., Toraldo, G., *et al.* 2002. Increased production of IL-7 uncouples bone formation from bone resorption during ER deficiency. *J. Clin. Invest.* **110**: 1643–1650.

373. Whyte, M.P. 2006. The long and the short of bone therapy. *N Engl J Med* **354**: 860-863.

374. Whyte, M.P., *et al.* 2000. Expansile skeletal hyperphosphatasia: a new familial metabolic bone disease. *J. Bone. Miner. Res.* **15**: 2320–2344.

375. Whyte, M.P., Hughes, A.E. 2002. Expansile skeletal hyperphosphatasia is caused by a 15-base pair tandem duplication in TNFRSF11A encoding RANK and is allelic to familial expansile osteolysis. *J. Bone. Miner. Res.* **17**: 26–29.

376. Widler, L., Jaeggi, K.A., Glatt, M., Muller, K., Bachmann, R., Bisping, W., Born, A.R., Cortesi, R., Guiglia, G., Jeker, H., Klein, R., Ramaseier, U., Schmid, J., Schreiber, G., Seltenmeyer, Y., Green, J.R. 2002. Highly potent geminal bisphosphonates. From pamidronate disodium (aredia) to ostedronic Acid (zometa). *J Med Chem* **45**: 3721–3738.

377. Wilbur, D.S., Hamlin, D.K., Buhler, K.R., Pathare, P.M., Vessella, R.L., Stayton, P.S., Richard, T. 1998. Streptavidin in antibody pretargeting. 2. Evaluation of methods for decreasing localization of streptavidin to kidney while retaining its tumor binding capacity. *Bioconjugate Chem* **9**: 322-330.

378. Wiktor-Jedrzejczak, W., Bartocci, A., Ferrante, A.W. Jr., Ahmed-Ansari, A., Sell, K.W., Pollard, J.W., Stanley, E.R. 1990. Total absence of colony-

stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci USA **87**: 4828-4832.

379. Wilchek, M. and Bayer, E. A., eds. 1990. Avidin-Biotin Technology. Academic Press, Inc., San Diego, CA.

380. Williamson, E., Bilsborough, J.M., Viney, J.L. 2002. Regulation of mucosal dendritic cell function by receptor activator of NF-kappa B (RANK)/RANK ligand interactions: impact on tolerance induction. *J. Immunol.* **169:** 3606–3612.

381. Windahl, S.H., et al. 2001. Female estrogen receptor beta–/– mice are partially protected against age-related trabecular bone loss. *J. Bone Miner. Res.* **16**: 1388–1398.

382. Wittrant, Y., Theoleyre, S., Couillaud, S., Dunstan, C., Heymann, D.m Redini, F. 2004. Relevance of an in vitro osteoclastogenesis system to study receptor activator of NF-kB ligand and osteoprotegerin biological activities. Exptl Cell Res **293**: 292-301.

383. Wong, B.R., *et al.* 1997. TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell specific survival factor. *J. Exp. Med.* **186**: 2075–2080.

384. Wong, B.R., *et al.* 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.* **272**: 25190–25194.

385. Wong, B.R., et al. 1998. The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor. J. Biol. Chem. **273**: 28355–28359.

386. Wong, B.R., Josien, R., Choi, Y. 1999. TRANCE is a TNF family member that regulates dendritic cell and osteoclast function. J. Leukoc. Biol. **65**: 715–724.

387. World Health Organization. 2003. Prevention and management of osteoporosis. WHO Technical Report series 921. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_921.pdf. Accessed on May 15, 2009.

388. Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitters, M.J., Kriz, R.W., Hewick, R.M., Wang, E.A. 1988. Science **242**: 1528–1534.

389. Yagi, M., Miyamoto, T., Sawatani, Y., Iwamoto, K., Hosogane, N., Fujita, N., Morita, K., Ninomiya, K., Suzuki, T., Miyamoto, K., Oike, Y., Takeya, M., Toyama, Y., Suda, T. 2005. DC-STAMP is essential for cell– cell fusion in osteoclasts and foreign body giant cells. J Exp Med. **202**: 345–51.

390. Yamaki, M., Nakamura, H., Takahashi, N., Udagawa, N., Ozawa, H. 2005. Arch. Biochem.Biophys. **440**: 10–17.

391. Yang, M., Mallhot, G., Birnbaum, M.J., MacKay, C.A., Mason-Savas, A., Odgren, P. 2006. Expression of and role for ovarian cancer G-protein-coupled receptor 1 (OGR1) during osteoclastogenesis. J. Biol. Chem. **281** (33): 23598-23605.

392. Yasuda, H. *et al.* 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. USA* **95:** 3597–3602.

393. Yoo, S. D., Jun, H., Shin, B. S., Lee, H. S., Park, M. O., Deluca, P. P., and Lee, K. C. 2001. Pharmacokinetic disposition of polyethylene glycol-modified salmon calcitonins in rat. Chem Pharm Bull **48**:1921–1924.

394. Yoshida, H., Enomoto, H., Kawamura, K., Takenaga, K., Tanabe, M., Ohnuma, N., et al. 1998. Antitumor vaccine effect of irradiated murine

neuroblastoma cells producing interleukin-2 or granulocyte macrophage-colony stimulating factor. Int J Oncol **13**: 73–8.

395. Yoshida, H., Hayashi, S-I., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L.D., Nishikawa, S-I. 1990. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature **345**: 442-444.

396. Yumita, S., Nicholson, G. C., Rowe, D. J., Kent, G. N., Martin, T. J. 1991. Biphasic effect of calcitonin on tartrate-resistant acid phosphatase activity in isolated rat osteoclasts. J Bone Miner Res **6**: 591–591.

397. Zaidi, M., Brain, S. D., Tippins, J. R., DiMarzo, V., Moonga, B. S., Chambers, T. J., Morris, H. R., MacIntyre, I. 1990. Structure-activity relationship of human calcitonin gene-related peptide, Biochem J **269**: 775–780.

398. Zaidi, M., Chambers, T. J., Bevis, P. J. R., Beacham, J. L., Gaines Das, R. E., MacIntyre, I. 1988. Effects of peptides from the calcitonin genes on bone and bone cells. J Exp Physiol **73**: 471–485.

399. Zaidi, M., Datta, H. K., Moonga, B. S., MacIntyre, I. 1990. Evidence that the action of calcitonin on rat osteoclasts is mediated by two G-proteins acting via separate post-receptor pathways. J Endocrinol **126**: 473–481.

400. Zaidi, M., Datta, H.K., Patchell, A., Moonga, B., MacIntyre, I. 1989. Biochem. Biophys. Res. Commun. **163**: 1461–1465.

401. Zaidi, M., Inzerillo, A.M., Moonga, B.S., Bevis, P.J.R., Huang, C.L.-H. 2002. Forty years of calcitonin- where are we now? A tribute to the work of Iain Macintyre, FRS. Bone **30**(5): 655-663.

402. Zandberg, H., Moen, T. C., and Baas, P. D. 1995.Cooperation of the 5' and 3' processing sites as well as intron and exon sequences in calcitonin exon recognition. Nucl Acids Res **23**: 248–255.

403. Zenger, S., Hollberg, K., Ljusberg, J., Nogård, M., Ek-Rylnder, B., Kiviranta, R., Andersson, G. 2007. Bone **41**: 820-832.

404. Zhang, M., Sakahara, H., Yao, Z., Saga, T., Nakamoto, Y., Sato, N., Nakada, H., Yamashina, I., and Konishi, J. 1997. Intravenous Avidin Chase Improved Localization of Radiolabeled Streptavidin in Intraperitoneal Xenograft Pretargeted with Biotinylated Antibody. *Nucl. Med. Biol. 24*, 61-64.

405. Zhao, H., Kitaura, H., Sands, M.S., Ross, F.P., Teitelbaum, S.L, Novack, D.V. 2005. Critical role of beta3 integrin in experimental postmenopausal osteoporosis. J Bone Miner Res **20**: 2116–2123

406. Zhao, H., Parikka, V., Laitala-Leinonen, T., Väänänen, H.K. 2001. Downregulation of small GTPase Rab7 impairs osteoclast polarization and bone resorption. J. Biol. Chem. **276**: 39295-39302.

407. Zhou, H., Kartsogiannis, V., Hu, Y.S., Elliott, J., Quinn, J.M., McKinstry, W.J., Gillespie, M.T., Ng, K.W. 2001. J. Biol. Chem. **276**: 14916–14923.

408. Zhou, H., Kartsogiannis, V., Quinn, J.M., Ly, C., Gange, C., Elliott, J., Ng, K.W., Gillespie, M.T. 2002. J. Biol. Chem. **277**: 48808–48815.

409. Zou, W., Reeve, J.L., Liu, Y., Teitelbaum, S.L., Ross, P. 2008. Dap12 couples c-Fms activation to the osteoclast cytoskeleton by recruitment of Syk. 2008. Molecular Cell **31** (3): 422-431.